



1-31-00

A

Customer No. 20350
 TOWNSEND and TOWNSEND and CREW LLP
 Two Embarcadero Center, 8th Floor
 San Francisco, California 94111-3834
 (415) 576-0200

Attorney Docket No. 0140580-009810

ASSISTANT COMMISSIONER FOR PATENTS
 BOX PATENT APPLICATION
 Washington, D.C. 20231

"Express Mail" Label No. EL394876928US
 Date of Deposit: January 28, 2000

I hereby certify that this is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above, addressed to:

Assistant Commissioner for Patents
 Washington, D.C. 20231

By:
 John Fonteyn

Sir:

Transmitted herewith for filing under 37 CFR 1.53(b) is the

- ☒ patent application of
- ☐ continuation patent application of
- ☐ divisional patent application of
- ☐ continuation-in-part patent application of

Inventor(s)/Applicant Identifier: **Martin A. Cheever and Dirk Gheysen**

For: **HER-2/NEU FUSION PROTEINS**

- ☒ This application claims priority from each of the following Application Nos./filing dates:
60/117,976, filed 01/29/99
 the disclosure(s) of which is (are) incorporated by reference.
- ☐ Please amend this application by adding the following before the first sentence: "This application is a ☐ continuation ☐ continuation-in-part of and claims the benefit of U.S. Provisional Application No. 60/_____, filed _____, the disclosure of which is incorporated by reference."

Enclosed are:

- ☒ Title Page
- ☒ 80 sheet(s) of specification
- ☒ 11 sheet(s) of claims
- ☒ 32 sheet(s) of ☐ formal ☒ informal drawing(s).
- ☐ An assignment of the invention to _____
- ☐ A ☐ signed ☐ unsigned Declaration & Power of Attorney
- ☒ A ☐ signed ☒ unsigned Declaration.
- ☒ Abstract

In view of the Unsigned Declaration as filed with this application and pursuant to 37 CFR §1.53(f), Applicant requests deferral of the filing fee until submission of the Missing Parts of Application.

DO NOT CHARGE THE FILING FEE AT THIS TIME.

Annette S. Parent
 Reg No.: 42,058
 Attorneys for Applicant

Telephone:
 (415) 576-0200

Facsimile:
 (415) 576-0300



PATENT APPLICATION
HER-2/NEU FUSION PROTEINS

Inventor(s):

Martin A. Cheever, a citizen of the United States, residing at
6210 SE 22nd
Mercer Island, WA 98040

Dirk Gheysen, a citizen of Belgium, residing at
89, rue de l'Institut
B-1330 Rixensart
Belgium

Assignee:

Corixa Corporation

SmithKline Beecham

Entity:

Large

HER-2/NEU FUSION PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to the U.S. Provisional Application
5 No. 60/117,976, filed January 29, 1999, herein incorporated by reference in its entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

10

FIELD OF THE INVENTION

The present invention is generally directed to HER-2/neu fusion proteins,
nucleic acid molecules encoding HER-2/neu fusion proteins, viral vectors expressing
HER-2/neu fusion proteins, and pharmaceutical compositions (*e.g.*, vaccines) comprising
15 the HER-2/neu fusion proteins and/or nucleic acid molecules encoding the HER-2/neu
fusion proteins. The present invention is also directed to methods of treating or
preventing cancer by eliciting or enhancing an immune response to the HER-2/neu
protein, including for uses in the treatment of malignancies associated with the HER-
2/neu oncogene.

20

BACKGROUND OF THE INVENTION

Despite enormous investments of financial and human resources, cancer
remains one of the major causes of death. For example, cancer is the leading cause of
death in women between the ages of 35 and 74. Breast cancer is the most common
25 malignancy in women and the incidence for developing breast cancer is on the rise. It is
estimated that one in nine women will be diagnosed with the disease. Standard
approaches to cure breast cancer have centered around a combination of surgery,
radiation and chemotherapy. These approaches have resulted in some dramatic successes
in certain malignancies. However, breast cancer is most often incurable, when diagnosed
30 beyond a certain stage. Alternative approaches to early diagnosis and therapy are
necessary.

A common characteristic of malignancies is uncontrolled cell growth. Cancer cells appear to undergo a process of transformation from the normal phenotype to a malignant phenotype capable of autonomous growth. Amplification and overexpression of somatic cell genes is considered to be a common primary event that results in the transformation of normal cells to malignant cells. The malignant phenotypic characteristics encoded by the oncogenic genes are passed on during cell division to the progeny of the transformed cells.

At least forty oncogenes operative in malignant cells and responsible for, or associated with, transformation have been identified. These oncogenes have been classified into different groups based on the putative function or location of their gene products, such as the protein expressed by the oncogene.

Oncogenes are believed to be essential for certain aspects of normal cellular physiology. In this regard, the HER-2/neu oncogene appears to be a member of the tyrosine kinase family of receptor-like glycoproteins, and shares a high degree of identity with the epidermal growth factor receptor (EGFR). HER-2/neu presumably plays a role in cell growth and/or differentiation. HER-2/neu appears to induce malignancies through quantitative mechanisms that result from increased or deregulated expression of an essentially normal gene product.

The p185 glycoprotein is the protein product of the HER-2/neu oncogene. The HER-2/neu gene is amplified and p185 is overexpressed in a variety of cancers including breast, ovarian, colon, lung and prostate cancer. p185 is related to malignant transformation, and is found in 50-60% of ductal *in situ* carcinomas, in 20-40% of invasive breast cancers, and in a substantial fraction of adenocarcinomas arising in the ovaries, prostate, colon and lung. HER-2/neu expression is intimately associated not only with malignant phenotype, but also with the aggressiveness of the malignancy. HER-2/neu overexpression is correlated with a poor prognosis in both breast and ovarian cancers.

p185 is a transmembrane protein with a predicted relative molecular mass of 185 kD that is about 1255 amino acids in length. p185 has an extracellular domain (ECD) of about 645 amino acids with at least a 40% identity to EGFR, a highly hydrophobic transmembrane domain, and a carboxy terminal intracellular domain (ICD) of about 580 amino acids with at least a 80% identity to EGFR.

There is a need for anti-cancer vaccines that can target a malignancy with which the HER-2/neu oncogene is associated, and for compositions and methods that can

elicit and enhance an immune response to the HER-2/neu gene. The present invention is directed to these, as well as other, important ends.

SUMMARY OF THE INVENTION

5 The present invention provides HER-2/neu p185 fusion proteins, nucleic acid molecules that encode HER-2/neu fusion proteins, and viral vectors that comprise polynucleotide sequences encoding HER-2/neu fusion proteins, for uses that include the immunization of warm-blooded animals against malignancies with which the HER-2/neu oncogene is associated. Fusion proteins or nucleic acid molecules according to the
10 invention may be present in compositions that include a pharmaceutically acceptable carrier or diluent, e.g., an oil-in water emulsion, and optionally include one or more additional active ingredients, such as an immunostimulatory substance, e.g., SBAS-2, 3D-MPL, QS21, or a combination of 3D-MPL and QS21. The compositions of the invention are useful as, and can be in the form of vaccines. The fusion proteins, nucleic acid
15 molecules, viral vectors, pharmaceutical compositions and/or vaccines may be administered on a one-time basis (*e.g.*, for an individual with an elevated risk of acquiring or reacquiring a malignancy or when a malignancy is suspected) or on a periodic basis (*e.g.*, for an individual with an elevated risk of acquiring or reacquiring a malignancy or when a malignancy is suspected). Compounds or compositions of the present invention
20 are useful in treating one or more existing tumors, or preventing tumor occurrence or reoccurrence, in warm-blooded animals including humans.

 The present invention also provides methods for inhibiting or preventing the development of a cancer in a patient by eliciting or enhancing and immune response to the HER-2/neu protein, comprising administering to a patient a pharmaceutical
25 composition or vaccine as recited above. The patient may be afflicted with, *e.g.*, breast, ovarian, colon, lung or prostate cancer, in which case the methods provide treatment for the disease, or a patient considered at risk for such a disease may be treated prophylactically. In one embodiment, the administration of the pharmaceutical composition or vaccine comprises transfecting cells of a warm-blooded animal *ex vivo*
30 with a nucleic acid molecule of the invention or infecting cells of a warm-blooded animal *ex vivo* with a viral vector comprising a nucleic acid molecule of the invention, and subsequently delivering the transfected or infected cells to the warm-blooded animal.

 The present invention further provides, within other aspects, methods for removing tumor cells from a biological sample, comprising contacting a biological

sample with T cells that specifically react with a HER-2/neu fusion protein, wherein the step of contacting is performed under conditions and for a time sufficient to permit the removal of cells expressing the protein from the sample. Within related aspects, methods are provided for inhibiting the development of a cancer in a patient, comprising
5 administering to a patient a biological sample treated as described *supra*.

In another embodiment, methods are provided for stimulating and/or expanding T cells specific for a HER-2/neu fusion protein, comprising contacting T cells with one or more of: (i) a fusion protein as described above; (ii) a polynucleotide encoding such a fusion protein; and/or (iii) an antigen presenting cell that expresses such
10 a fusion protein; under conditions and for a time sufficient to permit the stimulation and/or expansion of T cells. Isolated T cell populations comprising T cells prepared as described above are also provided. Within further aspects, the present invention provides methods for inhibiting the development of a cancer in a patient, comprising administering to a patient an effective amount of a T cell population as described above.

In yet another embodiment, the present invention further provides methods for inhibiting the development of a cancer in a patient, comprising the steps of: (a) incubating CD4⁺ and/or CD8⁺ T cells isolated from a patient with one or more of: (i) a HER-2/neu fusion protein; (ii) a polynucleotide encoding such a fusion protein; and (iii) an antigen-presenting cell that expresses such a fusion protein; and (b) administering to
20 the patient an effective amount of the proliferated T cells, thereby inhibiting the development of a cancer in the patient. Proliferated cells may, but need not, be cloned prior to administration to the patient.

Finally, the invention provides a method of making a HER-2/neu fusion protein, the method comprising the steps of (a) introducing into a cell an expression
25 vector comprising a polynucleotide encoding a HER-2/neu fusion protein, (b) culturing the transfected cell; and (c) purifying the expressed protein. In a preferred embodiment, the cell is a CHO cell. In another preferred embodiment, the cell is cultured in suspension, under serum-free conditions. In yet another embodiment the expressed protein is purified by a two-step procedure, comprising an anion exchange
30 chromatography on Q sepharose High Performance Columns, and a hydrophobic chromatography on Phenyl Sepharose 6 Fast Flow low substitution.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All

references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 shows a map of the pFLAGCMV-1/ICD expression plasmid, which has a size of 6.7 kb.

 Figure 2 shows a map of the pFLAGCMV-1/KD expression plasmid, which has a size of 5.7 kb.

 Figure 3 shows a map of the pFLAGCMV-1/PD expression plasmid,
10 which has a size of 5.7 kb.

 Figure 4 shows that the HER-2/neu phosphorylation domain was secreted into the culture media, and that the HER-2/neu intracellular domain and HER-2/neu kinase domain were not secreted into the culture media, as described in Example 3.

 Figure 5 shows a map of the pcDNA3.1/hygro/ECD-PD expression vector,
15 which has a size of 8.3 kb.

 Figure 6 illustrates the results of the expression of the ECD-PD fusion protein in HEK-293 and CHO cells, and shows that the fusion protein was secreted into the culture media, as described in Example 4.

 Figure 7 shows the full length amino acid sequence of the human HER-
20 2/neu protein (SEQ ID NO:1).

 Figure 8 shows the full length amino acid sequence of the rat HER-2/neu protein (SEQ ID NO:2). The kinase domain spans the region from amino acid 721 to amino acid 998, inclusively.

 Figure 9 shows the amino acid sequence of the extracellular HER-2/neu
25 protein (SEQ ID NO:3).

 Figure 10 shows the amino acid sequence of the phosphorylation domain (PD) of the human HER-2/neu protein (SEQ ID NO:4).

 Figure 11 shows the amino acid sequence of a preferred portion of the phosphorylation domain (Δ PD) of the human HER-2/neu protein (SEQ ID NO:5).

30 Figure 12 shows the amino acid sequence of a fusion protein comprising the extracellular domain (ECD) and the phosphorylation domain (PD) of the human HER-2/neu protein (SEQ ID NO:6).

Figure 13 shows the amino acid sequence of a fusion protein comprising the extracellular domain (ECD) and a preferred portion of the phosphorylation domain (Δ PD) of the human HER-2/neu protein (SEQ ID NO:7).

Figure 14 shows the amino acid sequence of the extracellular domain (ECD) of the rat HER-2/neu protein (SEQ ID NO:8).

Figure 15 shows the full length nucleotide sequence (SEQ ID NO:9) of a DNA molecule encoding the human HER-2/neu protein. This full length nucleotide sequence is described in WO 96/30514, the disclosure of which is incorporated by reference herein in its entirety.

Figure 16 shows the full length nucleotide sequence (SEQ ID NO:10) of a DNA molecule encoding the rat HER-2/neu protein. This full length nucleotide sequence is described by Bargmann *et al.* (1986) *Nature*, 319:226-30, and GENBANK/X03362, the disclosures of which are incorporated by reference herein in their entirety.

Figure 17 illustrates the results of an ELISA assay for Herceptin binding to different ECD-PD fusion proteins produced either in mammalian cells or in *E. coli*. The fusion proteins produced in *E. coli* are in frame with a C- or N-terminal 6 x histidine tag (noted C-His tag and N-His tag, respectively).

Figure 18 shows a comparison of HER-2/neu ECD-PD fusion protein expression in CHO-K1, grown in suspension under serum-free conditions, and in *Pichia* cells.

Figure 19 shows the nucleotide sequence of mouse Her-2/neu.

Figure 20 shows the amino acid sequence of mouse Her-2/neu.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

INTRODUCTION

The present invention is directed to compounds and compositions capable of modulating, preferably eliciting or enhancing, immunity to the protein product of HER-2/neu oncogene expression, including for malignancies in a warm-blooded animal where an amplified HER-2/neu gene with a malignancy does not require that the protein expression product of the gene be present on the tumor. For example, overexpression of the gene may be involved with initiation and early stages of tumor formation, but the protein expression may subsequently be reduced or absent. The present invention may be used to elicit or enhance an effective immune response to convert a HER-2/neu positive

tumor to HER-2/neu negative, in addition to preventing the establishment of HER-2/neu positive tumors and provoking the regression of existing HER-2/neu positive tumors.

The following abbreviations are used throughout the specification: "ECD" refers to the extracellular domain, "ICD" refers to the intracellular domain, "PD" refers to the phosphorylation domain (*i.e.*, the domain that is phosphorylated) that is within the intracellular domain, "ΔPD" refers to a fragment of the phosphorylation domain that is within the phosphorylation domain, and "KD" refers to the kinase domain that is within the intracellular domain. The product of expression of the HER-2/neu gene is referred to herein as the "HER-2/neu protein," also known and referred to as "p185" or "c-erbB2."

10

DEFINITIONS

The "HER-2/neu ECD-ICD fusion protein," also referred to herein as "ECD-ICD" or "ECD-ICD fusion protein," refers to a fusion protein (or fragments thereof) comprising the extracellular domain (or fragments thereof) and the intracellular domain (or fragments thereof) of the HER-2/neu protein. As used herein, the ECD-ICD fusion protein does not include a substantial portion of the HER-2/neu transmembrane domain, and preferably does not include any of the HER-2/neu transmembrane domain.

The "HER-2/neu ECD-PD fusion protein," also referred to as "ECD-PD" or "ECD-PD fusion protein," or the "HER-2/neu ECD-ΔPD fusion protein," also referred to as "ECD-ΔPD" or "ECD-ΔPD fusion protein," refer to fusion proteins (or fragments thereof) comprising the extracellular domain (or fragments thereof) and phosphorylation domain (or fragments thereof, *e.g.*, ΔPD) of the HER-2/neu protein. The ECD-PD and ECD-ΔPD fusion proteins do not include a substantial portion of the HER-2/neu transmembrane domain, and preferably do not include any of the HER-2/neu transmembrane domain.

25

The terms "HER-2/neu ECD-ICD fusion protein" and "HER-2/neu ECD-PD fusion protein" and their related terms are also understood to refer to fragments thereof, homologs thereof and functional equivalents thereof (collectively referred to as "variants"), such as those in which one or more amino acids is inserted, deleted or replaced by other amino acid(s) or non-amino acid(s) which, in preferred embodiments of the invention, either (i) increase the elicitation or enhancement of an immune response as compared to the HER-2/neu protein, or (ii) do not substantially affect elicitation or enhancement of an immune response as compared to the HER-2/neu protein (*e.g.*, variant

stimulates a response by helper T cells or cytotoxic T cells or stimulates the production of antibodies). Specific, non-limiting, examples of variants including exemplary fragments, homologs and functional equivalents of the HER-2/neu ECD-ICD fusion protein and HER-2/neu ECD-PD fusion protein are described in more detail herein. Variants can be

5 “substantially identical” or “substantially similar” to a fusion protein comprising native polypeptide components, and retain the ability to stimulate an immune response.

A “fusion protein” refers to a protein having at least two polypeptides covalently linked, in which one polypeptide comes from one protein sequence or domain and the other polypeptide comes from another protein sequence or domain. The

10 polypeptides can be linked either directly or via a covalent linker, *e.g.*, an amino acid linker, such as a polyglycine linker, or another type of chemical linker, *e.g.*, a carbohydrate linker, a lipid linker, a fatty acid linker, a polyether linker, *e.g.*, PEG, *etc.* (See, *e.g.*, Hermanson, *Bioconjugate techniques* (1996)). The polypeptides forming the fusion protein are typically linked C-terminus to N-terminus, although they can also be

15 linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. The polypeptides of the fusion protein can be in any order. The term “fusion protein” also refers to conservatively modified variants, polymorphic variants, alleles, mutant, subsequences and interspecies homologues of the polypeptides that make up the fusion protein. Fusion proteins may be produced by covalently linking a chain of amino acids

20 from one protein sequence to a chain of amino acids from another protein sequence, *e.g.*, by preparing a recombinant polynucleotide contiguously encoding the fusion protein. Fusion proteins can comprise 2, 3, 4 or more different chains of amino acids from the same or different species. The different chains of amino acids in a fusion protein may be directly spliced together or may be indirectly spliced together via a chemical linking

25 group or an amino acid linking group. The fusion protein may optionally comprise other components, as described in more detail herein.

The term “protein” is used herein interchangeably with “polypeptide” and “peptide.”

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and

30 polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include,

without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.* (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka *et al.* (1985) *J. Biol. Chem.* 260:2605-2608; Rossolini *et al.* (1994) *Mol. Cell. Probes* 8:91-98). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

A polynucleotide sequence comprising a fusion protein of the invention hybridizes under stringent conditions to each of the nucleotide sequences encoding each individual polypeptide of the fusion protein. The polynucleotide sequences encoding the individual polypeptides of the fusion polypeptide therefore include conservatively modified variants, polymorphic variants, alleles, mutants, subsequences, and interspecies homologs.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 25% sequence identity. Alternatively, percent identity can be any integer from 25% to 100%. More preferred embodiments include at least: 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% or higher, compared to a reference sequence using the programs described herein; preferably BLAST using standard parameters, as described below. One of skill will recognize that these values can be appropriately

adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. "Substantial identity" of amino acid sequences for these purposes normally means sequence identity of at least 40%. Preferred percent identity of polypeptides can be any integer from 40% to 100%. More preferred embodiments include at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes.

Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, aspartic acid-glutamic acid, and asparagine-glutamine.

Optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math.* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1977) *Nuc. Acids Res.* 25:3389-3402 and Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0)

and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other, or to a third nucleic acid, under moderately, and preferably highly, stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

For the purpose of the invention, suitable "moderately stringent conditions" include, for example, prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridizing at 50°C-65°C, 5X SSC overnight, followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC (containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention.

"Proliferation of T cells," as described herein, includes the multiplication of T cells as well as the stimulation of T cells leading to multiplication, *i.e.*, the initiation of events leading to mitosis and mitosis itself. Methods for detecting proliferation of T cells are discussed below.

FUSION PROTEINS OF THE INVENTION

A. Intra- and extracellular domains of the HER-2/neu protein

HER-2/neu protein was selected as a target for anti-cancer vaccines based on observations of HER-2/neu as described above. One of the major obstacles to this approach is the difficulty of isolating a sufficient amount of HER-2/neu protein. One attempt to address this problem was to express the ECD and ICD separately in mammalian cells. The ECD expressed at a high level as a secreted protein, *i.e.*, about 20 mg of the ECD protein were purified from one liter mouse cell culture. However, the level of expression of ICD was low, *i.e.*, only about 0.2 mg of the ICD protein were purified from one liter HEK-293 cells. In addition, the resulting ICD protein was very labile in cell lysate, creating numerous unexpected problems for purification of useful quantities.

As discussed above, HER-2/neu is an oncogenic self protein, and immunological tolerance to self proteins can dampen the immune response. The level of immunological tolerance to different portions of any particular protein may depend upon whether the expressed portion of the protein resides within or without the cell membrane. The ECD resides on the cell surface and is shed. By contrast, the ICD and portions thereof reside inside the cell and are not shed. The ECD readily comes in contact with the body's immune system, whereas the ICD and portions thereof are relatively sequestered from the body's immune system. As a result, the level of immunologic tolerance to ECD is greater than that to the ICD portion of the HER-2/neu protein. Thus, for vaccines according to the present invention, ICD protein and ICD peptides, and variants thereof, including PD proteins and PD peptides, induce a relatively greater level of immune response than ECD protein and ECD peptides.

Although the ICD and its variants are more immunogenic than the ECD and its variants, antibody to ECD and its variants is beneficial and possibly desirable. The ECD resides at the cell surface whereas the ICD and portions thereof are not secreted and are sequestered inside the cell. Thus, antibody responses to the ECD can have a greater therapeutic benefit, and accordingly are preferred according to the invention. The ECD by itself is not very immunogenic. Since the ICD (including the PD and Δ PD) is more immunogenic than the ECD, the ECD-ICD fusion protein and/or the ECD-PD fusion protein is more immunogenic than the ECD alone. The ECD-ICD fusion protein and/or the ECD-PD fusion protein is expected to be more effective for inducing antibody to the ECD than is the ECD alone, and is a preferred embodiment of the invention.

In the present invention, the ECD or its variants is combined, linked or fused (either directly or indirectly) with the ICD or its variants, preferably with the PD or its variants. The ECD provides the structural conformation for inducing antibodies that react with HER-2/neu protein at the cell surface, while the ICD or PD increases the immunogenicity of the ECD. The combination is surprisingly more effective for inducing an immune response to the ECD than is the ECD alone.

The ECD or portions of the ECD can be combined with the ICD or its variants, including portions of the ICD or with the PD or its variants, including portions of the PD (*e.g.*, the Δ PD). The ECD of the present invention is preferably a human, a rat or a mouse ECD. The human ECD is set forth in Fig. 9 and as SEQ ID NO:3. The rat ECD is set forth in Fig. 14 and as SEQ ID NO:8.

The ICD of the present invention is preferably a human, a rat or a mouse ICD. The human ICD is set forth in Fig. 7 and SEQ ID NO:1 as inclusively spanning the region of Lys 676 to Val 1255. The rat ICD is set forth in Fig. 8 and SEQ ID NO:2 as inclusively spanning the region of Lys 677 to Val 1256.

The PD of the present invention is preferably a human, a rat or a mouse PD. The human PD is set forth in Fig. 10 and as SEQ ID NO:4. The human PD may be the human Δ PD, which is set forth in Fig. 11 and as SEQ ID NO:5. The rat PD is shown in Fig. 8 and SEQ ID NO:2 as inclusively spanning the region of Gln 991 to Val 1256. The rat PD may be the rat Δ PD, which is shown in Fig. 8 and SEQ ID NO:2 as inclusively spanning the region of Gln 991 to Arg 1049.

In one embodiment, a human ECD can be fused with either (i) a human ICD or a rat ICD or (ii) a human PD or Δ PD, or a rat PD or Δ PD. In another

embodiment, a rat ECD can be fused with either (i) a human ICD or a rat ICD or (ii) a human PD or Δ PD, or a rat PD or Δ PD.

The HER-2/neu PD is 268 amino acids in length, is intracellular, and can be phosphorylated by protein tyrosine kinases. This region shares no identity with the corresponding part of other tyrosine kinase receptors. Thus, the specificity and uniqueness of this domain makes it particularly preferred for use as a tumor vaccine. However, the expression of this domain alone in bacteria and mammalian cells is problematic. For example, the resultant PD protein is very labile and is not appropriate for large scale production. In one embodiment, this invention has solved such problems by fusing all or part of the intracellular domain or the phosphorylation domain to all or part of the HER-2/neu extracellular domain. The ECD-ICD fusion proteins and the ECD-PD fusion proteins of the invention are soluble, are secreted and are stable in culture media. This system can provide large quantities of intracellular domain or phosphorylation domain protein for cancer vaccine development, preferably breast cancer vaccine development, but will be useful for vaccines against any cancer characterized by HER-2/neu expression. In addition to allowing increased expression of the intracellular domain or phosphorylation domain, or variants thereof, as a fusion protein with the extracellular domain or its variants, the ECD-ICD and ECD-PD fusion proteins provide for an improved vaccine formulation.

The PD was secreted by introducing a secretion signal sequence preceding the N-terminus of the PD to yield a soluble, secreted recombinant protein. The secretion process is preferred since the recombinant proteins accumulate in the culture media. Because the protein is not associated with the intracellular proteins, proteolysis is limited. The protein should be purified more easily and economically.

As described in Example 3, pFLAGCMV-1 expression plasmid (Kodak) was used to determine which region of the HER-2/neu intracellular domain was able to be secreted. The proteins were expressed as fusion proteins with a preprotrypsin secretion signal and FLAG-Tag at their N-terminus. HEK-293 cells were transfected with such constructs, and cell and culture media were assayed for FLAG-Tag fusion proteins by western blot with FLAG-Tag M2 antibody as probe. The results in Fig. 4 demonstrate that neither full length ICD nor KD was secreted, but that the PD was soluble and was secreted and detected in the culture media. The results indicate that the full length

structure ICD or KD protein did not result in secretion, that is in passage of the protein through the cell membrane.

As described in Example 4, since the ECD has a secretion signal sequence and can be expressed well as a secreted protein, the ECD was used as a fusion partner for PD. The ECD-PD fusion protein was expressed in HEK-293 cells. The secretion of soluble ECD-PD fusion protein was determined by ELISA assay with HER-2/neu ECD-specific antibodies, followed by western blot with HER-2/neu PD-specific antibodies. As shown in Fig. 6, the soluble ECD-PD expressed in HEK-293 and was secreted into the culture medium.

B. Immunogenicity of the fusion proteins of the invention

In a preferred embodiment, the present invention is directed to a fusion protein based on particular portions (e.g., HER-2/neu ECD-ICD fusion protein or HER-2/neu ECD-PD fusion protein) of the protein expression product of the HER-2/neu gene, which is capable of eliciting an antibody response and can be recognized by thymus-dependent lymphocytes ("T-cells"). Accordingly, the autochthonous immune T cell response can be used prophylactically or to treat malignancies in which HER-2/neu is or has been overexpressed. In another aspect, the present invention is directed to the use of nucleic acid molecules directing the expression of such ECD-ICD fusion proteins or ECD-PD fusion proteins, or their variants, alone or in a viral vector for immunization.

In general, CD4⁺ T cell populations are considered to function as helpers or inducers through the release of lymphokines when stimulated by a specific antigen; however, a subset of CD4⁺ cells can act as cytotoxic T lymphocytes (CTL). Similarly, CD8⁺ T cells are considered to function by directly lysing antigenic targets; however, under a variety of circumstances they can secrete lymphokines to provide helper or DTH function. Despite the potential of overlapping function, the phenotypic CD4 and CD8 markers are linked to the recognition of peptides bound to class I or class II MHC antigens. The recognition of antigen in the context of class I or class II MHC mandates that CD4⁺ and CD8⁺ T cells respond to different antigens or the same antigen presented under different circumstances. The binding of immunogenic peptides to class II MHC antigens most commonly occurs for antigens ingested by antigen presenting cells.

As disclosed within the present invention, an ECD-ICD fusion protein or an ECD-PD fusion protein of the protein expression product of the HER-2/neu oncogene is recognized by T cells. Circulating HER-2/neu ECD-ICD fusion protein or HER-2/neu

ECD-PD fusion protein is degraded to peptide fragments. Peptide fragments from the ECD-ICD fusion protein or ECD-PD fusion protein bind to major histocompatibility complex (MHC) antigens. By display of a peptide bound to MHC antigen on the cell surface and recognition by host T cells of the combination of peptide plus self MHC antigen, the HER-2/neu ECD-ICD or the HER-2/neu ECD-PD fusion protein (including that expressed on a malignant cell) will be immunogenic to T cells. The exquisite specificity of the T cell receptor enables individual T cells to discriminate between protein fragments which differ by a single amino acid residue.

During the immune response to a peptide fragment from the ECD-ICD fusion protein or the ECD-PD fusion protein, T cells expressing a T cell receptor with high affinity binding of the peptide-MHC complex will bind to the peptide-MHC complex and thereby become activated and induced to proliferate. In the first encounter with a peptide, small numbers of immune T cells will secrete lymphokines, proliferate and differentiate into effector and memory T cells. The primary immune response will occur *in vivo* but has been difficult to detect *in vitro*. A subsequent encounter with the same antigen by the memory T cells will lead to a faster and more intense immune response. The secondary response will occur either *in vivo* or *in vitro*. The *in vitro* response is easily gauged by measuring the degree of proliferation, the degree of cytokine production, or the generation of cytolytic activity of the T cell population re-exposed in the antigen. Substantial proliferation of the T cell population in response to a particular antigen is considered to be indicative of prior exposure to or priming with the antigen.

C. Fusion proteins of the invention

In one embodiment, the compounds of the present invention comprise HER-2/neu ECD-ICD fusion proteins or variants, or polynucleotides that encode such ECD-ICD fusion proteins. Preferably, the nucleic acid molecules are DNA molecules. In the HER-2/neu ECD-ICD fusion proteins of the invention, the ECD and ICD polypeptide components can be directly fused or fused via a linker, *e.g.*, an amino acid linker or another type of chemical linker. In a preferred embodiment, the ECD-ICD fusion proteins of the present invention comprise all or a portion of the HER-2/neu ECD fused directly to all or a portion of the HER-2/neu ICD.

In additional embodiments, the size of the ECD in the ECD-ICD fusion protein can be altered by sequentially removing anywhere from 1 to about 100 amino acids from the carboxyl terminus of the ECD, preferably about 100 amino acids.

Similarly, the size of the ICD in the ECD-ICD fusion protein can be altered by sequentially removing anywhere from 1 to about 100 amino acids from the N-terminus and/or the C-terminus of the ICD. The resulting variant forms may be selected on the basis of their antigenicity and/or immunogenicity using appropriate screening methods as described in the literature and herein, for use according to the invention.

In another embodiment, the compounds of the present invention comprise HER-2/neu ECD-PD fusion proteins, or variants, or nucleic acid molecules that encode such ECD-PD fusion proteins. In one embodiment, the HER-2/neu ECD is fused to the HER-2/neu Δ PD. Preferably, the nucleic acid molecules are DNA molecules. In the HER-2/neu ECD-PD fusion proteins of the invention, the ECD and PD or Δ PD polypeptide components can be directly fused or fused via a linker, *e.g.*, a peptide linker. In a preferred embodiment, the ECD-PD fusion protein of the present invention comprises the HER-2/neu ECD fused directly to the HER-2/neu PD or to the HER-2/neu Δ PD. Here and throughout the specification, a preferred embodiment of the fusion proteins of the invention is the HER-2/neu PD fusion protein.

In another embodiment, the size of the ECD in the ECD-PD fusion protein is altered by sequentially removing anywhere from 1 to about 100 amino acids from the carboxyl terminus of the ECD, preferably about 100 amino acids. Similarly, the size of the PD in the ECD-PD fusion protein can be altered by sequentially removing amino acids from the N-terminus of the PD. Again, the preferred embodiment is PD. Other variant forms may be selected on the basis of their antigenicity and/or immunogenicity using appropriate screening methods as described in the literature and herein, for use according to the invention.

Table 1 shows that removing 100 amino acids from the carboxyl terminus of the ECD does not have any impact on the expression level and stability of the ECD-PD fusion protein.

Table 1: ECD_{truncated}-PD Summary

| Clone I.D. | area of deletion (bp/aa) | # of bp | # of aa | relative expression |
|------------|--------------------------|---------|---------|---------------------|
| A5 | 1655-1882 / 552-628 | 228 | 76 | *** |
| B4 | 1660-1866 / 554-622 | 207 | 69 | ** |
| B9 | 1595-1891 / 532-631 | 297 | 99 | *** |
| C2 | 1681-1902 / 561-634 | 222 | 74 | * |
| C7 | 1612-1902 / 538-634 | 291 | 97 | ***** |
| F10 | 1634-1951 / 545-651 | 318 | 106 | **** |
| ECD-PD WT | - | - | - | * |

5 Variants of the ECD-ICD fusion protein and the ECD-PD fusion protein also include various structural forms of the native ECD-ICD fusion protein and ECD-PD fusion protein, respectively. Due to the presence of ionizable amino and carboxyl groups, for example, a HER-2/neu ECD-ICD or ECD-PD fusion protein can be in the form of an acidic or basic salt, or may be in a neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

10 Other variants within the scope of the invention include ECD-ICD fusion proteins or ECD-PD fusion proteins in which the primary amino acid structure native HER-2/neu ECD-ICD protein or native HER-2/neu ECD-PD protein, respectively, is modified by forming covalent or aggregative conjugates with other peptides or polypeptides, or chemical moieties such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives may be prepared, for example, by linking
15 particular functional groups to amino acid side chains or at the N- or C-terminus.

The present invention also includes HER-2/neu ECD-ICD fusion proteins and HER-2/neu ECD-PD fusion proteins with or without glycosylation. ECD-ICD fusion proteins and ECD-PD fusion proteins expressed in yeast or mammalian expression systems may be similar to, or slightly different in molecular weight and glycosylation
20 pattern from, the native molecules, depending upon the expression system. Expression of DNA encoding polypeptides in bacteria such as *E. coli* typically provides non-glycosylated molecules. N-glycosylation sites of eukaryotic proteins are characterized by the amino acid triplet Asn-A₁-Z, wherein A₁ is any amino acid except Pro, and Z is Ser or Thr. Variants of HER-2/neu ECD-ICD or ECD-PD fusion proteins having inactivated N-

glycosylation sites can be produced by techniques known to one skilled in the art, such as oligonucleotide synthesis and ligation or site-specific mutagenesis techniques, and are within the scope of the invention. Alternatively, N-linked glycosylation sites can be added to a HER-2/neu ECD-ICD or ECD-PD fusion protein.

5 The ECD-ICD fusion proteins of the present invention, which will be understood to include variants, include any possible combination between human and non-human polypeptides. Non-human polypeptides comprise polypeptides from any mammal, such as, *e.g.*, rat, mouse, guinea pig, horse, cow, pig, sheep, dog, *etc.* In one embodiment, the ECD-ICD fusion proteins include:

10 (i) human ECD - human ICD fusion proteins, such as those formed by linking the human ECD of Fig. 9 (SEQ ID NO:3) with the human ICD, which is the amino acid sequence inclusively spanning Lys 676 to Val 1255, as shown in Fig. 7 (SEQ ID NO:1), with or without a chemical and/or amino acid linking group, and variants thereof;

15 (ii) rat ECD - rat ICD fusion proteins, such as those formed by linking the rat ECD of Fig. 14 (SEQ ID NO:8) with the rat ICD, which is the amino acid sequence inclusively spanning Lys 677 to Val 1256, as shown in Fig. 8 (SEQ ID NO:2), with or without a chemical and/or amino acid linking group, and variants thereof;

20 (iii) human ECD - rat ICD fusion proteins, such as those formed by linking the human ECD shown in Fig. 9 (SEQ ID NO:3) with the rat ICD, which is the amino acid sequence inclusively spanning Lys 677 to Val 1256, as shown in Fig. 8 (SEQ ID NO:2), with or without a chemical and/or amino acid linking group, and variants thereof; and

25 (iv) rat ECD - human ICD fusion proteins, such as those formed by linking the rat ECD, as shown in Fig. 14 (SEQ ID NO:8), with the human ICD, which is the amino acid sequence inclusively spanning Lys 676 to Val 1255, as shown in Fig. 7 (SEQ ID NO:1), with or without a chemical and/or amino acid linking group, and variants thereof.

30 Any variants of the ECD-ICD fusion proteins of the present invention are included as embodiments of the present invention. In one embodiment, such variants are substantially identical or substantially similar to the native HER-2/neu ECD-ICD protein and retain the ability to stimulate an immune response. Human DNA sequences that encode the ECD protein are shown, for example, in Fig. 15 (SEQ ID NO:9) as inclusively spanning nucleotide 1 to nucleotide 1959. Human DNA sequences that encode the ICD

protein are shown, for example, in Fig. 15 (SEQ ID NO:9) as inclusively spanning nucleotide 2026 to nucleotide 3765. The effect of any sequence modification on the ability of a HER-2/neu ECD-ICD protein to produce an immune response may be readily determined, for example, by analyzing the ability of the mutated HER-2/neu ECD-ICD protein to induce a T cell response using, for example, the methods described herein, or by analyzing the ability of the mutated HER-2/neu ECD-ICD protein to produce antibodies.

The ECD-PD fusion proteins of the present invention, which will be understood to include variants, include any possible combination between human and non-human polypeptides. Non-human polypeptides comprise, *e.g.*, rat, mouse, guinea pig, horse, cow, pig, sheep, dog, *etc.* In one embodiment, the ECD-PD fusion proteins include:

(i) human ECD - human PD fusion proteins, such as shown in Fig. 12 (SEQ ID NO:6) and variants thereof, including fusion proteins formed by linking the human ECD of Fig. 9 (SEQ ID NO:3) with the human PD of Fig. 10 (SEQ ID NO:4) with or without a chemical and/or amino acid linking group, and variants thereof;

(ii) rat ECD - rat PD fusion proteins, such as those formed by linking the rat ECD of Fig. 14 (SEQ ID NO:8) with the rat PD, which is the amino acid sequence inclusively spanning Gln 991 to Val 1256, as shown in Fig. 8 (SEQ ID NO:2), with or without a chemical and/or amino acid linking group, and variants thereof;

(iii) human ECD - rat PD fusion proteins, such as those formed by linking the human ECD shown in Fig. 9 (SEQ ID NO:3) with the rat PD, which is the amino acid sequence inclusively spanning Gln 991 to Val 1256, as shown in Fig. 8 (SEQ ID NO:2), with or without a chemical and/or amino acid linking group, and variants thereof; and

(iv) rat ECD - human PD fusion proteins, such as those formed by linking the rat ECD, as shown in Fig. 14 (SEQ ID NO:8), with the human PD, as shown in Fig. 10 (SEQ ID NO:4), with or without a chemical and/or amino acid linking group, and variants thereof.

Any variants of the ECD-PD fusion proteins of the present invention are included as embodiments of the present invention. In one embodiment, such variants are substantially identical or substantially similar to the native HER-2/neu ECD-PD protein and retain the ability to stimulate an immune response. Human DNA sequences that encode the ECD protein are shown, for example, in Fig. 15 (SEQ ID NO:9) as inclusively spanning nucleotide 1 to nucleotide 1959. Human DNA sequences that encode the PD

protein are shown, for example, in Fig. 15 (SEQ ID NO:9) as inclusively spanning nucleotide 2968 to nucleotide 3765. The effect of any sequence modification on the ability of a HER-2/neu ECD-PD protein to produce an immune response may be readily determined, for example, by analyzing the ability of the mutated HER-2/neu ECD-PD protein to induce a T cell response using, for example, the methods described herein, or by analyzing the ability of the mutated HER-2/neu ECD-PD protein to produce antibodies.

In another embodiment, the ECD-PD fusion proteins are ECD- Δ PD fusion proteins of the present invention, which will be understood to include variants, including any possible combination between human and non-human polypeptides. Non-human polypeptides comprise, *e.g.*, rat, mouse, guinea pig, horse, cow, pig, sheep, dog, *etc.* In one embodiment, the ECD- Δ PD fusion proteins include:

(i) human ECD - human Δ PD fusion proteins, such as shown in Fig. 13 (SEQ ID NO:7) and variants thereof, including fusion proteins formed by linking the human ECD of Fig. 9 (SEQ ID NO:3) with the human Δ PD of Fig. 11 (SEQ ID NO:5) with or without a chemical and/or amino acid linking group, and variants thereof;

(ii) rat ECD - rat Δ PD fusion proteins, such as those formed by linking the rat ECD of Fig. 14 (SEQ ID NO:8) with the rat Δ PD, which is the amino acid sequence inclusively spanning Gln 991 to Arg 1049, as shown in Fig. 8 (SEQ ID NO:2), with or without a chemical and/or amino acid linking group, and variants thereof;

(iii) human ECD - rat Δ PD fusion proteins, such as those formed by linking the human ECD shown in Fig. 9 (SEQ ID NO:3) with the rat Δ PD, which is the amino acid sequence inclusively spanning Gln 991 to Arg 1049, as shown in Fig. 8 (SEQ ID NO:2), with or without a chemical and/or amino acid linking group, and variants thereof; and

(iv) rat ECD - human Δ PD fusion proteins, such as those formed by linking the rat ECD, as shown in Fig. 14 (SEQ ID NO:8), with the human Δ PD, as shown in Fig. 11 (SEQ ID NO:5), with or without a chemical and/or amino acid linking group, and variants thereof.

Any variants of the ECD- Δ PD fusion proteins of the present invention are included as embodiments of the present invention. In one embodiment, such variants are substantially identical or substantially similar to the native HER-2/neu ECD- Δ PD protein and retain the ability to stimulate an immune response. Human DNA sequences that

encode the ECD protein are shown, for example, in Fig. 15 (SEQ ID NO:9) as inclusively spanning nucleotide 1 to nucleotide 1959. Human DNA sequences that encode the Δ PD protein are shown, for example, in Fig. 15 (SEQ ID NO:9) as inclusively spanning nucleotide 2968 to nucleotide 3144. The effect of any sequence modification on the ability of a HER-2/neu ECD- Δ PD protein to produce an immune response may be readily determined, for example, by analyzing the ability of the mutated HER-2/neu ECD- Δ PD protein to induce a T cell response using, for example, the methods described herein, or by analyzing the ability of the mutated HER-2/neu ECD- Δ PD protein to produce antibodies.

In a preferred embodiment, the HER-2/neu ECD-PD fusion proteins of the invention are ECD-PD fusion proteins.

Within certain specific embodiments, the fusion proteins of the invention may comprise a fusion partner, such as, *e.g.*, an immunological fusion partner or an expression enhancer. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the fusion protein (an expression enhancer) at higher yields than the recombinant fusion protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the fusion protein or to enable the fusion protein to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the fusion protein.

Also provided are fusion proteins that comprise a fusion polypeptide as described herein together with an unrelated immunogenic protein. Preferably, the immunogenic protein is capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see, e.g., Stoute et al. (1997) New Engl. J. Med. 336:86-91*).

In other embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium *Haemophilus influenza B* (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.*, the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the fusion protein with additional exogenous T-cell epitopes and to increase the expression level in

E. coli (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the fusion protein to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemagglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene* 43:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (*see Biotechnology* 10:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion protein. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

In a preferred embodiment, the fusion proteins of the present invention further comprise a fusion partner. Preferred fusion partners include, but are not limited to, *e.g.*, Ra12 or LeIF. In particular, the invention provides materials and methods for using a Ra12 or a LeIF sequence as a fusion partner to facilitate the stable and high yield expression of recombinant fusion polypeptides or break tolerance.

Ra12 is a 14 kD C-terminal fragment of the *M. tuberculosis* MTB32A coding sequence, which is expressed at high levels on its own and remains soluble throughout the purification process. LeIF is a *Leishmania* antigen which is homologous to the eukaryotic ribosomal protein eIF and which is capable of stimulating a Th1 and/or CTL immune response (*See, e.g.*, U.S. Patent Nos. 5,876,966, 5,876,735, and 5,879,687). The present invention utilizes these properties of the Ra12 and LeIF polypeptides and provides recombinant nucleic acid molecules, expression vectors, host cells, and methods for stable and high yield expression of fusion polypeptides comprising, in addition to the HER-2/neu fusion polypeptides, a Ra12 or LeIF polypeptide. Recombinant nucleic acids, which encode a fusion polypeptide comprising a Ra12 or LeIF polypeptide and a fusion protein of interest, can be readily constructed by conventional genetic engineering

techniques. Recombinant nucleic acids are constructed so that, preferably, the fusion partner polynucleotide sequence is located 5' to a selected fusion protein sequence. It may also be appropriate to place an fusion partner polynucleotide sequence 3' to the polynucleotide sequence of the fusion protein of interest or to insert the polynucleotide sequence of the fusion protein into a site within an fusion partner polynucleotide sequence. In addition, any suitable polynucleotide that encodes a fusion partner or a portion or other variant thereof as described herein can be used in constructing recombinant fusion nucleic acids of the present invention.

Nucleic acids encoding the fusion partner polypeptides of this invention can be prepared by any suitable method known in the art. Exemplary methods include cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.* (1979) *Meth. Enzymol.* 68: 90-99; the phosphodiester method of Brown *et al.* (1979) *Meth. Enzymol.* 68: 109-151; the diethylphosphoramidite method of Beaucage *et al.* (1981) *Tetra. Lett.*, 22: 1859-1862; and the solid support method of U.S. Patent No. 4,458,066.

Recombinant nucleic acids that encode a fusion polypeptide comprising an fusion partner polypeptide and a selected fusion protein can be prepared using any methods known in the art. As described above, recombinant nucleic acids are constructed so that, preferably, the fusion partner polynucleotide sequence is located 5' to the polynucleotide sequence encoding the fusion protein of interest. The fusion partner and fusion protein polynucleotide sequences can also be modified to facilitate their fusion and subsequent expression.

The recombinant nucleic acids can further comprise other nucleotide sequences such as sequences that encode affinity tags to facilitate protein purification protocol.

D. Variants of the fusion proteins of the invention

CD4⁺ T cells generally recognize antigens that have been external to the tumor cells. By contrast, under normal circumstances, binding of peptides to class I MHC occurs only for proteins present in the cytosol and synthesized by the target itself, proteins in the external environment are excluded. An exception to this is the binding of exogenous peptides with a precise class I binding motif which are present outside the cell in high concentration. Thus, CD4⁺ and CD8⁺ T cells have broadly different functions and tend to recognize different antigens as a reflection of where the antigens normally reside.

Another way to make amino acid substitutions to produce variants of the present invention is to identify and replace amino acids in T cell motifs with potential to bind to class II MHC molecules (for CD4⁺ T cell response) or class I MHC molecules (for CD8⁺ T cell response). Peptide segments (of a HER-2/neu ECD-ICD or ECD-PD fusion protein) with a motif with theoretical potential to bind to class II MHC molecules may be identified by computer analysis. For example, a protein sequence analysis package, *T Sites*, that incorporates several computer algorithms designed to distinguish potential sites for T cell recognition can be used (Feller *et al.* (1991) *Nature*, 349:720-721). Two searching algorithms are used: (1) the AMPHI algorithm described by Margalit (Feller *et al.* (1991) *Nature*, 349:720-721; Margalit *et al.* (1987) *J. Immunol.*, 138:2213-2229) identifies epitope motifs according to alpha-helical periodicity and amphipathicity; (2) the Rothbard and Taylor algorithm identifies epitope motifs according to charge and polarity pattern (Rothbard *et al.* (1988) *EMBO*, 7:93-100). Segments with both motifs are most appropriate for binding to class II MHC molecules. CD8⁺ T cells recognize peptides bound to class I MHC molecules. Parker *et al.* (1994) *J. Immunol.*, 152:163 have determined that peptides binding to particular MHC molecules share discernible sequence motifs. A peptide motif for binding in the groove of HLA-A2.1 has been defined by Edman degradation of peptides stripped from HLA-A2.1 molecules of a cultured cell line (Table 2, from Falk *et al.* (1991) *Nature*, 351:290-296). The method identified the typical or average HLA-A2.1 binding peptide as being 9 amino acids in length with dominant anchor residues occurring at positions 2 (L) and 9 (V). Commonly occurring strong binding residues have been identified at positions 2 (M), 4 (E,K), 6 (V), and 8 (K). The identified motif represents the average of many binding peptides.

Table 2: The HLA-A2.1 Restricted Motif

| | Amino Acid Position | | | | | | | | | Point Assignment |
|---------------------------------|----------------------------|---|----------------------------|------------------|---------------------------------|-------------|-------------|--------|---|------------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Dominant Binding Anchor Residue | | L | | | | | | | V | +3 |
| Strong Binding Residue | | M | | E K | | V | | K | | +2 |
| Weak Binding Residue | I L F K M Y | | A Y F P M S | G P D T | I K Y N G V H | I L T | A Y H | E S | L | +1 |

The derived peptide motif as currently defined is not particularly stringent. Some HLA-A2.1 binding peptides do not contain both dominant anchor residues and the amino acids flanking the dominant anchor residues play major roles in allowing or disallowing binding. Not every peptide with the current described binding motif will bind, and some peptides without the motif will bind. However, the current motif is valid enough to allow identification of some peptides capable of binding. Of note, all MHC molecules and respective motifs place 6 amino acids between the dominant anchor amino acids at residues 2 and 9.

Following identification of peptide motifs within HER-2/neu ECD-ICD or ECD-PD fusion proteins, amino acid substitutions can be made conservatively or non-conservatively. The latter type of substitutions are intended to produce an improved ECD-ICD or ECD-PD fusion protein or polypeptide that is more potent and/or more broadly cross-reactive. An example of a more potent protein or peptide is one that binds with higher affinity to the same MHC molecule as the natural protein or polypeptide, without affecting recognition by T cells specific for natural protein or polypeptide. An example of a polypeptide with broader cross-reactivity is one that induces more broadly cross-reactive immune responses (*i.e.*, binds to a greater range of MHC molecules) than

natural polypeptide. Similarly, one or more amino acids residing between peptide motifs and having a spacer function (*e.g.*, do not interact with a MHC molecule or T cell receptor) can be substituted conservatively or non-conservatively. It will be evident to one of ordinary skill in the art that polypeptides containing one or more amino acid
5 substitutions can be tested for beneficial or adverse immunological interactions by a variety of assays, including those described herein for the ability to stimulate T cell recognition.

Variants within the scope of this invention can also, or alternatively, contain other modifications, including the deletion or addition of amino acids, that have
10 minimal influence on the desired immunological properties of the polypeptide, as described *supra*. It will be appreciated by one of ordinary skill in the art that truncated forms or non-native extended forms of a HER-2/neu ECD-ICD or ECD-PD fusion protein can be used, provided the desired immunological properties are at least roughly equivalent to that of full length, native HER-2/neu ECD-ICD or ECD-PD fusion protein.
15 Cysteine residues may be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present.

20 **PREPARING THE FUSION PROTEINS OF THE INVENTION**

A. Polynucleotides encoding fusion proteins

The invention relates to isolated or purified polynucleotides that encode the HER-2/neu fusion proteins. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the fusion protein can be used to
25 generate recombinant molecules which direct the expression of the fusion protein.

In order to clone full-length coding sequences or homologous variants to generate the HER-2/neu fusion polynucleotides, labeled DNA probes designed from any portion of the HER-2/neu nucleotide sequences or their complements may be used to screen a genomic or cDNA library, to identify the coding sequence of each individual
30 component of the fusion protein. The Her-2/neu nucleotide sequences can be from any suitable mammal, *e.g.*, rat, mouse, horse, cow, pig, sheep, dog, *etc.*

In one embodiment, the Her-2/neu sequence is from a human, a rat, or a mouse. The sequence of mouse Her-2/neu is shown in Figure 19 (SEQ ID NO:11). Mouse Her-2/neu can also be amplified from mouse brain RNA using the following

primers: 5' primer: CCATGGAGCTGGCGGCCTGGTGCCGTTG (SEQ ID NO:12) and
3' primer: GGCCTTCTGGTTCATACTGGCACATCCAGGC (SEQ ID NO:13). The
mouse Her-2/neu amino acid sequence is shown in Figure 20 (SEQ ID NO:14). A variant
amino acid sequence for mouse Her-2/neu is described in Nagata *et al.*, *J. Immunol.*

5 159:1336-1343 (1997).

Such clones may be isolated by screening an appropriate expression library
for clones that express full length HER-2/neu protein. The library preparation and screen
may generally be performed using methods known to one of ordinary skill in the art, such
as methods described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*,
10 Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1989), which is incorporated
herein by reference. Briefly, a bacteriophage expression library may be plated and
transferred to filters. The filters may then be incubated with a detection reagent. In the
context of this invention, a "detection reagent" is any compound capable of binding to
HER-2/neu protein, which may then be detected by any of a variety of means known to
15 one of ordinary skill in the art. Typical detection reagents contain a "binding agent," such
as Protein A, Protein G, IgG or a lectin, coupled to a reporter group. Preferred reporter
groups include enzymes, substrates, cofactors, inhibitors, dyes, radionuclides,
luminescent groups, fluorescent groups and biotin. More preferably, the reporter group is
horseradish peroxidase, which may be detected by incubation with a substrate such as
20 tetramethylbenzidine or 2,2'-azino-di-3-ethylbenz-thiazoline sulfonic acid. Plaques
containing genomic or cDNA sequences that express HER-2/neu protein are isolated and
purified by techniques known to one of ordinary skill in the art. Appropriate methods
may be found, for example, in Sambrook *et al.*, *Molecular Cloning: A Laboratory
Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1989).

25 Isolation of coding sequences may also be carried out by the polymerase
chain reactions (PCR) using two degenerate oligonucleotide primer pools designed on the
basis of the coding sequences disclosed herein. The desired nucleic acids can also be
cloned using other well known amplification techniques. Examples of protocols
sufficient to direct persons of skill through *in vitro* amplification methods, including PCR,
30 ligase chain reaction (LCR), Q β -replicase amplification and other RNA polymerase
mediated techniques are found in Sambrook, and Ausubel, as well as U.S. Patent No.
4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds. 1990);
Arnheim & Levinson *C&EN* pp. 36-47 (October 1, 1990); *The Journal of NIH Research*,
3:81-94 (1991); Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173; Guatelli *et al.*

(1990) *Proc. Natl. Acad. Sci. USA* 87:1874; Lomell *et al.* (1989) *J. Clin. Chem.* 35:1826; Landegren *et al.* (1988) *Science* 241:1077-1080; Van Brunt (1990) *Biotechnology* 8:291-294; Wu *et al.* (1989) *Gene* 4:560; and Barringer *et al.* (1990) *Gene* 89:117. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S.

5 Pat. No. 5,426,039. Suitable primers for use in the amplification of the nucleic acids of the invention can be designed based on the sequences provided herein.

In accordance with the invention, a polynucleotide of the invention which encodes a fusion protein, fragments thereof, or functional equivalents thereof may be used to generate recombinant nucleic acid molecules that direct the expression of the fusion
10 protein, fragments thereof, or functional equivalents thereof, in appropriate host cells. The fusion polypeptide products encoded by such polynucleotides may be altered by molecular manipulation of the coding sequence.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence,
15 may be used in the practice of the invention for the expression of the fusion polypeptides. Such DNA sequences include those which are capable of hybridizing to the coding sequences or their complements disclosed herein under low, moderate or high stringency conditions described herein.

Altered nucleotide sequences which may be used in accordance with the
20 invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues, which result in a silent change thus producing a functionally equivalent antigenic epitope. Such conservative amino acid substitutions may be made on the basis
25 of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine, histidine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: glycine, asparagine, glutamine,
30 serine, threonine and tyrosine; and amino acids with nonpolar head groups include alanine, valine, isoleucine, leucine, phenylalanine, proline, methionine and tryptophan.

The nucleotide sequences of the invention may be engineered in order to alter the fusion protein coding sequence for a variety of ends, including but not limited to, alterations which modify processing and expression of the gene product. For example,

mutations may be introduced using techniques which are well known in the art, *e.g.*, to insert or delete restriction sites, to alter glycosylation patterns, phosphorylation, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions, to facilitate further *in vitro* modification, *etc.* One of skill will

5 recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include, *e.g.*, site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to chemical mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-
10 known techniques (*see, e.g.*, Gilman *et al.* (1979) *Gene* 8:81-97, Hutchinson, *et al.* (1978) *J. Biol. Chem.* 253:6551; Roberts *et al.* (1987) *Nature* 328: 731-734). Preferably, the manipulations do not destroy immunogenicity of the fusion polypeptides.

In one embodiment of the invention, the coding sequence of a fusion protein could be synthesized in whole or in part, using chemical methods well known in
15 the art (*see, e.g.*, Caruthers *et al.* (1980) *Nuc. Acids Res. Symp. Ser.* 7:215-233; Crea *et al.* (1980) *Nuc. Acids Res.* 9(10):2331; Matteucci *et al.* (1980) *Tetrahedron Letter* 21:719 (1980); and Chow *et al.* (1981) *Nuc. Acids Res.* 9(12):2807-2817).

B. Polypeptide synthesis

20 Alternatively, the fusion polypeptide itself can be produced using chemical methods to synthesize an amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, such as, *e.g.*, the Merrifield solid phase synthesis method, in which amino acids are sequentially added to a growing chain of amino acids (*see*, Merrifield (1963) *J. Am. Chem. Soc.*, 85:2149-2146). Equipment for
25 automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer Biosystems, Inc. (Foster City, CA), and may generally be operated according to the manufacturer's instructions. The synthesized peptides can then be cleaved from the resin, and purified, *e.g.*, by preparative high performance liquid chromatography (*see* Creighton, *Proteins Structures and Molecular Principles*, 50-60
30 (1983)). The composition of the synthetic fusion polypeptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; *see* Creighton, *Proteins, Structures and Molecular Principles*, pp. 34-49 (1983)).

In addition, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the sequence. Non-classical amino acids

include, but are not limited to, the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, α -methyl amino acids, $N\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

C. Linking groups

In another embodiment, the polypeptides of the fusion protein, e.g., ECD and ICD or the ECD and PD, are joined via a linking group. The linking group can be a chemical crosslinking agent, including, for example, succinimidyl-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC). The linking group can also be an additional amino acid sequence(s), including, for example, a polyglycine linking group.

In a specific embodiment, the coding sequences of each polypeptide in the fusion protein are directly joined at their amino- or carboxy-terminus via a peptide bond in any order.

Alternatively, an amino acid linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such an amino acid linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.* (1985) *Gene* 40:39-46; Murphy *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:8258-8262; U.S. Patent Nos. 4,935,233 and 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and

prevent steric interference.

Other chemical linkers include carbohydrate linkers, lipid linkers, fatty acid linkers, polyether linkers, *e.g.*, PEG, *etc.* (See, *e.g.*, Hermanson, *Bioconjugate Techniques* (1996)).

5

D. Additional polypeptides

As described *supra*, the fusion protein may be linked to one or more additional polypeptides. For example, the fusion polypeptide may be linked to one or more copies of one of the two polypeptides of the fusion protein. Alternatively, the fusion protein may be linked to an additional heterologous polypeptide, such as, *e.g.*, Ra12 or LeIF, as described *supra*. The fusion polypeptide may also be fused to an affinity tag for ease of purification upon expression. For example, multiple histidine residues encoded by the tag allow the use of metal chelate affinity chromatography methods for the purification of fusion polypeptides. Other examples of affinity tag molecules include, *e.g.*, Strep-tag, PinPoint, maltose binding protein, glutathione S-transferase, *etc.* (see, *e.g.*, Glick & Pasternak, *Molecular Biotechnology Principles and Applications of Recombinant DNA* (2nd ed. 1999)).

In one embodiment, the fusion polypeptide is optionally linked to a lipid moiety, such as mycolic acid, lipoaribdomanin ("LAMs"), or trehalose derivatives.

As described above, in one embodiment, such a fusion protein is produced by recombinant expression of a nucleic acid encoding the fusion protein. Such a fusion product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the product by methods known in the art. Alternatively, such a product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Coding sequences for other molecules such as a cytokine or an adjuvant can be added to the fusion polynucleotide as well.

E. Sequence modifications

Variants of the fusion proteins of the invention that retain the ability to stimulate an immune response may generally be identified by modifying the sequence in one or more of the aspects described above and assaying the resulting fusion protein for the ability to stimulate an immune response, *e.g.*, a T cell response or an antibody response. For example, such assays may generally be performed by contacting T cells

with the modified fusion protein and assaying the response. Naturally occurring variants of the individual polypeptide components of the fusion protein may also be isolated by, for example, screening an appropriate cDNA or genomic library with a DNA sequence encoding each individual polypeptide or a variant thereof.

5 The above-described sequence modifications may be introduced using standard recombinant techniques or by automated synthesis of the modified fusion protein. For example, mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting
10 reconstructed sequence encodes an analogue having the desired amino acid insertion, substitution, or deletion.

 Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be used to provide a gene in which particular codons are altered according to the substitution, deletion, or insertion required. Exemplary methods of making the
15 alterations set forth above are described by Walder *et al.* (1986) *Gene*, 42:133; Bauer *et al.* (1985) *Gene*, 37:73; Craik (1985) *BioTechniques*, January:12-19; Smith *et al.*, *Genetic Engineering: Principles and Methods*, Plenum Press (1981); and U.S. Patent Nos. 4,518,584 and 4,737,462.

 Mutations in nucleotide sequences constructed for expression of such
20 HER-2/neu fusion proteins must, of course, preserve the reading frame of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, which would adversely affect translation of the mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order
25 to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed HER-2/neu fusion protein mutants screened for the desired activity. Not all mutations in a nucleotide sequence which encodes a HER-2/neu fusion protein will be expressed in the final product. For example, nucleotide substitutions may be made to enhance expression, primarily to avoid
30 secondary structure loops in the transcribed mRNA (*see, e.g.*, European Patent Application 75,444A), or to provide codons that are more readily translated by the selected host, such as the well-known *E. coli* preference codons for *E. coli* expression.

F. Expression vectors

The Her-2/neu fusion proteins, and variants thereof, of the present invention, are preferably produced by recombinant DNA methods. Such methods include inserting a DNA sequence encoding a HER-2/neu fusion protein into a recombinant

5 expression vector and expressing the DNA sequence in a recombinant microbial, mammalian, fungal or insect cell expression system under conditions promoting expression and, preferably, secretion of the fusion protein. DNA sequences encoding the Her-2/neu fusion proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to
10 provide a synthetic gene which is capable of being inserted in a recombinant expression vector and expressed in a recombinant transcriptional unit.

Recombinant expression vectors contain a DNA sequence encoding a Her-2/neu fusion protein operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, fungal, microbial, viral or insect genes. Such
15 regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. An origin of replication and a selectable marker to facilitate recognition of transformants may additionally be incorporated.

20 DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to
25 a coding sequence if it is positioned so as to permit translation. Generally, "operably linked" means contiguous and, in the case of secretory leaders, in reading frame. DNA sequences encoding HER-2/neu fusion proteins which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA.

30 Expression vectors for bacterial use may comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, *e.g.*, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), pGEM1 (Promega Biotec, Madison, WI), pET28b (Novagen) and pPDM (a

modified pET28b, Corixa). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar *et al.* (1977) *Gene*, 2:95). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. Promoters commonly used in recombinant microbial expression vectors include the β -lactamase (penicillinase) and lactose promoter system (Chang *et al.* (1978) *Nature*, 275:615; and Goeddel *et al.* (1979) *Nature*, 281:544), the tryptophan (trp) promoter system (Goeddel *et al.* (1980) *Nucl. Acids Res.*, 8:4057; and European Patent Application 36,776) and the tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p.412 (1982)). A particularly useful bacterial expression system uses the phage λ P_L promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Suitable promoter sequences in yeast vectors include the promoters for alcohol oxidase, metallothionein, 3-phosphoglycerate kinase (Hitzeman *et al.* (1980) *J. Biol. Chem.*, 255:2073) or other glycolytic enzymes (Hess *et al.* (1968) *J. Adv. Enzyme Reg.*, 7:149; and Holland *et al.* (1978) *Biochem.*, 17:4900), such as enolase, glyceraldehyde- 3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in European Patent Application No. 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α -factor secretion leader. The ADH2 promoter has been described by Russell *et al.* (1982) *J. Biol. Chem.*, 258:2674 and Beier *et al.* (1982) *Nature*, 300:724. The yeast α -factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed (*see, e.g.*, Kurjan *et al.* (1982) *Cell*, 30:933; and Bitter *et al.* (1984) *Proc. Natl. Acad. Sci. USA*, 81:5330. The leader sequence may be

modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived, *e.g.*, from polyoma, adenovirus 2, simian virus (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, the SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers *et al.* (1978) *Nature*, 273:113). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* II site located in the viral origin of replication is included. Further, viral genomic promoter, control and/or signal sequences may be used, provided such control sequences are compatible with the host cell chosen. Exemplary vectors can be constructed as disclosed by Okayama *et al.* (1983) *Mol. Cell. Biol.*, 3:280.

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman *et al.* (1986) *Mol. Immunol.*, 23:935. A suitable eukaryotic vector for expression of the fusion proteins of the invention is pDC406 (McMahan *et al.* (1991) *EMBO J.*, 10:2821 which includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). Other vectors include pDC409 and pDC410, which are derived from pDC406. pDC410 was derived from pDC406 by substituting the EBV origin of replication with sequences encoding the SV40 large T antigen. pDC409 differs from pDC406 in that a *Bgl* II restriction site outside of the multiple cloning site has been deleted, making the *Bgl* II site within the multiple cloning site unique. Any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in mammalian cells are also suitable.

In addition to the transcriptional and translational control sequences, some expression systems have markers that provide gene amplification, such as neomycin, thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase.

A useful cell line that allows for episomal replication of expression vectors, such as pDC406 and pDC409, which contain the EBV origin of replication, is CV-1/EBNA (ATCC CRL 10478). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-I (EBNA-1) and constitutively expresses EBNA-1 driven from human CMV immediate-early enhancer/promoter.

Preferred vectors for expression in mammalian cultured cells include pFLAGCMV-1 (Kodak), pcDNA3.1/hyg (Invitrogen) and pEE14-GS (CellTech).

G. Host cells

Transformed host cells are cells which have been transformed or transfected with expression vectors constructed using recombinant DNA techniques and which contain sequences encoding HER-2/neu fusion proteins of the present invention. Transformed host cells may express the desired HER-2/neu fusion proteins, but host cells transformed for purposes of cloning or amplifying HER-2/neu DNA do not need to express the HER-2/neu fusion proteins. Expressed Her-2/neu fusion proteins will preferably be secreted into the culture medium or supernatant, depending on the DNA selected. One skilled in the art will appreciate that if Her-2/neu fusion proteins are secreted into the culture supernatant, then they are also soluble in the culture supernatant.

Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used to introduce the expression vector. These include the use of reagents such as Superfect (Qiagen), liposomes, calcium phosphate transfection, polybrene, protoplast fusion, electroporation, microinjection, plasmid vectors, viral vectors, biolistic particle acceleration (the gene gun), or any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g., Sambrook et al., supra*).

Suitable host cells for expression of recombinant proteins include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *Bacilli*. Higher eukaryotic cells include established cell lines of insect or mammalian origin as described below. Cell-free translation systems could also be used to produce

HER-2/neu fusion proteins using RNAs derived from DNA constructs. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, by Pouwels *et al.*, *Cloning Vectors: A Laboratory Manual*, Elsevier, New York (1985).

5 Prokaryotic expression hosts may be used for expression of HER-2/neu fusion proteins that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, *e.g.*, a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure
10 amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli* (*e.g.*, BL21 (DE3) CodonPlus *E. coli*), *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although other hosts may also be used.

Recombinant HER-2/neu fusion proteins may also be expressed in yeast
15 hosts such as *P. pastoris*. Yeast of other genera, such as *Saccharomyces*, *Schizosaccharomyces* or *Kluyveromyces*, may also be used. Expression in *Pichia* is achieved by ligation of the gene to be expressed into a bacterial shuttle vector (*e.g.*, the pPICZ series from Invitrogen Co.), transformation of the yeast with this vector and chromosomal integration into the alcohol oxidase (AOX) locus of the yeast genome.
20 Selection for recombinant yeast is then performed using, *e.g.*, Zeocin (Invitrogen Co.) and protein expression is induced by the addition of methanol to the growth medium (Higgin *et al.*, "Pichia Protocols," *Methods in Molecular Biology*, Vol. 103, Humana Press (1998)). Suitable strains of *Pichia* for protein expression include, *e.g.*, the SMD1168 *Pichia* strain. Expression systems based on other methodologies, such as the ESP system
25 (Stratagene) may also be used.

Suitable yeast transformation protocols are known to one of skill in the art. An exemplary technique described by Hind *et al.*, *Proc. Natl. Acad. Sci. USA*, 75:1929 (1978), involves selecting for Trp⁺ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 mg/ml adenine and 20
30 mg/ml uracil. Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 mg/ml adenine and 80 mg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect (*e.g.*, *Spodoptera* or *Trichoplusia*) cell culture systems can also be used to express recombinant polypeptide. Baculovirus systems for production of heterologous polypeptides in insect cells are reviewed, for example, by Luckow *et al.* (1988) *BioTechnology*, 6:47. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (1981) *Cell*, 23:175, and other cell lines capable of expressing an appropriate vector including, *e.g.*, CV-1/EBNA (ATCC CRL 10478), L cells, C127, 3T3, Chinese hamster ovary (CHO), COS, NS-1, HeLa, Human embryonic Kidney Fibroblasts (HEK 293) and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements (*e.g.*, an origin of replication, a suitable promoter and/or an enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences) and 5' or 3' nontranslated sequences (*e.g.*, necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences). A preferred mammalian expression system is the Chinese hamster ovary (CHO) cell line.

H. Purification of the fusion proteins of the invention

Purified HER-2/neu fusion proteins may be prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts. For example, supernatants from systems which secrete recombinant polypeptide into culture media may be first concentrated using a commercially available protein concentration filter, such as, *e.g.*, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a counter structure protein (*i.e.*, a protein to which a HER-2/neu fusion protein binds in a specific interaction based on structure) or lectin or antibody molecule bound to a suitable support.

Alternatively, an anion exchange resin can be used, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose, polystyrene, sepharose or other types commonly used in protein purification. Alternatively, a cation exchange step can be used. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups, preferably sulfopropyl groups. Gel filtration chromatography also provides a means of purifying HER-2/neu fusion proteins. The fusion proteins of the

invention are preferably purified by anion exchange chromatography using, *e.g.*, monoQ columns or Q sepharose High Performance chromatography.

Affinity chromatography is another preferred method of purifying HER-2/neu fusion proteins. For example, monoclonal antibodies against the HER-2/neu fusion proteins may be useful in affinity chromatography purification, by using methods that are well-known in the art.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps using hydrophobic RP-HPLC media (*e.g.*, silica gel having pendant methyl or other aliphatic groups) may be used to further purify HER-2/neu fusion protein compositions. Some or all of the foregoing purification steps, in various combinations, can also be used to provide a homogeneous recombinant protein or polypeptide.

Recombinant HER-2/neu fusion proteins produced in bacterial culture may be by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid chromatography (HPLC) may be used for final purification steps. Microbial cells used in expression of recombinant HER-2/neu fusion proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express HER-2/neu fusion proteins as a secreted protein greatly simplifies purification. The secreted recombinant proteins resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal *et al.* (1984) *J. Chromatog.*, 296:171. This reference describes two sequential, reverse-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Preparations of HER-2/neu fusion proteins synthesized in recombinant cultures may contain non-HER-2/neu cell components, including proteins, in amounts and of a character which depend upon the purification steps taken to recover the HER-2/neu fusion proteins from the culture. These components are ordinarily of yeast, prokaryotic or non-human eukaryotic origin. Such preparations are typically free of other proteins which may be normally associated with the HER-2/neu protein as it is found in nature in its species of origin.

Automated synthesis provides an alternate method for preparing proteins and polypeptides of this invention. For example, any of the commercially available solid-

phase techniques may be used, such as, *e.g.*, the Merrifield solid phase synthesis method, in which amino acids are sequentially added to a growing amino acid chain. (*See*, Merrifield (1963) *J. Am. Chem. Soc.*, 85:2149-2146). Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied Biosystems, Inc. (Foster City, CA), and may generally be operated according to the manufacturer's instructions.

In general, polypeptides (including fusion proteins) and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

BINDING AGENTS

The present invention further provides agents, such as antibodies and antigen-binding fragments thereof, that specifically bind to a HER-2/neu fusion protein of the invention. As used herein, an antibody, or antigen-binding fragment thereof, is said to "specifically bind" to a HER-2/neu fusion protein if it reacts at a detectable level, *i.e.*, at least two fold over background signal (within, for example, an ELISA) with a HER-2/neu fusion protein, and does not react detectably with unrelated proteins under similar conditions. As used herein, "binding" refers to a noncovalent association between two separate molecules such that a complex is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind," in the context of the present invention, when the binding constant for complex formation exceeds about 10^3 l/mol. The binding constant maybe determined using methods well known in the art.

Binding agents may be further capable of differentiating between patients with and without a cancer, such as breast, ovarian, colon, lung or prostate cancer, using the representative assays provided herein. In other words, antibodies or other binding agents that bind to a HER-2/neu fusion protein will generate a signal indicating the

presence of a cancer in at least about 20% of patients with the disease, and will generate a negative signal indicating the absence of the disease in at least about 90% of individuals without the cancer. To determine whether a binding agent satisfies this requirement, biological samples (*e.g.*, blood, sera, plasma, urine and/or tumor biopsies) from patients
5 with and without a cancer (as determined using standard clinical tests) may be assayed as described herein for the presence of polypeptides that bind to the binding agent. It will be apparent that a statistically significant number of samples with and without the disease should be assayed. Each binding agent should satisfy the above criteria; however, those of ordinary skill in the art will recognize that binding agents may be used in combination
10 to improve sensitivity.

Any agent that satisfies the above requirements may be a binding agent. For example, a binding agent may be a ribosome, with or without a peptide component, an RNA molecule or a polypeptide. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Antibodies may be prepared by any of a
15 variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of
20 recombinant antibodies. In one technique, an immunogen comprising, *e.g.*, a fusion polypeptide or the sequence corresponding to the junction between the individual polypeptides of a fusion protein of interest (referred to as "junction region"), is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep or goats). In this step, the fusion protein of interest or the junction region of the fusion protein of the
25 invention may serve as the immunogen without modification. Alternatively, particularly for relatively short sequences, a superior immune response may be elicited if the sequence is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the
30 animals are bled periodically. Polyclonal antibodies specific for the fusion polypeptide may then be purified from such antisera by, for example, affinity chromatography using the fusion polypeptide coupled to a suitable solid support.

Polyclonal antibodies raised to a fusion protein of the invention can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive

with the fusion protein of interest and not with the individual polypeptide components of the fusion proteins. This selection may be achieved by subtracting out antibodies that cross-react with the individual polypeptide components of the fusion protein of interest.

Alternatively, antibodies that recognize each or all of the individual
5 polypeptide components of a fusion protein may be useful in the context of the present invention.

Monoclonal antibodies specific for an immunogenic fusion polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein (1976) *Eur. J. Immunol.* 6:511-519, and improvements thereto. Briefly, these methods involve
10 the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the fusion polypeptide of interest). Such cell lines may be produced, *e.g.*, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, *e.g.*, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion
15 techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are
20 observed. Single colonies are selected and their culture supernatants tested for binding activity against the fusion polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the
25 yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The fusion polypeptides of this invention may be used in the purification
30 process in, for example, an affinity chromatography step.

Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane,

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988) and digested by papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by affinity chromatography on protein A bead columns.

Monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y , ^{123}I , ^{125}I , ^{131}I , ^{186}Re , ^{188}Re , ^{211}At , and ^{212}Bi . Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, *Diphtheria* toxin, cholera toxin, gelonin, *Pseudomonas* exotoxin, *Shigella* toxin, and pokeweed antiviral protein.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, including, *e.g.*, U.S. Patent No. 4,671,958.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of

different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody.

- 10 Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

- A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as, *e.g.*, albumins (*e.g.*, U.S. Patent No. 4,507,234), peptides and polysaccharides such as, *e.g.*, aminodextran (*e.g.*, U.S. Patent No. 4,699,784). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562 discloses representative chelating compounds and their synthesis.

- A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration will be intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody used, the antigen density on the tumor, and the rate of clearance of the antibody.

Examples of available suitable antibodies to the fusion proteins of the invention include, but are not limited to, the 8029K rabbit polyclonal antibody, the mouse monoclonal c-neu-3 antibody (Calbiochem), and the mouse monoclonal Herceptin antibody (U.S. Patent 5,677,171). The monoclonal c-neu-3 antibody recognizes a

sequential epitope in the PD domain which is deleted (1242-1255 aa) in the ECD-ΔPD construct. The Herceptin antibody binds to a conformational epitope in the ECD domain.

T CELLS

5 Immunotherapeutic compositions may also, or alternatively, comprise T cells specific for a fusion protein of the present invention. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood of a patient, using a commercially available cell separation system (*see also* U.S. 10 Patent Nos. 5,240,856 and 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human mammals, cell lines or cultures.

T cells may be stimulated with a HER-2/neu fusion polypeptide, a polynucleotide encoding a HER-2/neu fusion protein and/or an antigen presenting cell 15 (APC) that expresses such a fusion polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the fusion polypeptide. Preferably, a HER-2/neu fusion polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of specific T cells.

20 T cells are considered to be specific for a HER-2/neu fusion polypeptide if the T cells kill target cells coated with the fusion polypeptide or expressing a polynucleotide encoding the fusion polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis 25 and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen *et al.* (1994) *Cancer Res.* 54:1065-1070. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.*, by pulse-labeling 30 cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Contact with a HER-2/neu fusion polypeptide (100 ng/ml - 100 µg/ml, preferably 200 ng/ml - 25 µg/ml) for 3 - 7 days should result in at least a two fold increase in proliferation of the T cells. Contact as described above for 2-3

hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN- γ) is indicative of T cell activation (*see* Coligan *et al.*, Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998)). T cells that have been activated in response to a
5 HER-2/neu fusion polypeptide, polynucleotide or fusion polypeptide-expressing APC may be CD4⁺ and/or CD8⁺. HER-2/neu fusion protein-specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient, or from a related or unrelated donor, and are administered to the patient following stimulation and expansion.

10 For therapeutic purposes, CD4⁺ or CD8⁺ T cells that proliferate in response to a HER-2/neu fusion polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to a HER-2/neu fusion polypeptide with or without the addition of T cell growth factors, such as
15 interleukin-2, and/or stimulator cells that synthesize a HER-2/neu fusion polypeptide. Alternatively, one or more T cells that proliferate in the presence of a HER-2/neu fusion protein can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution. Following expansion, the cells may be administered back to the patient as described, for example, by Chang *et al.* (1996) *Crit.*
20 *Rev. Oncol. Hematol.* 22:213.

PHARMACEUTICAL COMPOSITIONS AND VACCINES COMPRISING FUSION PROTEINS OF THE INVENTION

In another preferred embodiment, the present invention is directed to
25 compositions comprising HER-2/neu fusion proteins, or variants thereof, and HER-2/neu ICD proteins, or variants thereof. The fusion proteins are preferably ECD-ICD fusion proteins, ECD-PD fusion proteins, or variants thereof, of the present invention, as described in detail herein. The ICD protein is preferably the human ICD protein, which spans the region from Lys 676 to Val 1255, inclusively, as shown in Fig. 7 (SEQ ID
30 NO:1), or the rat ICD protein, which is the amino acid sequence inclusively spanning Lys 677 to Val 1256 as shown in Fig. 8 (SEQ ID NO:2). Alternatively, the HER-2/neu ICD protein can be any variant or portion of the ICD protein that is immunogenic or that provides enhanced immunogenicity to the composition. For example, the portion of the

ICD protein may be the HER-2/neu PD protein, as described herein, the HER-2/neu Δ PD protein, as described herein, the HER-2/neu KD protein, as described herein, or a HER-2/neu ICD protein where anywhere from 1 to 100 amino acids are sequentially removed from the N-terminus or C-terminus of the ICD protein. In addition, amino acid

- 5 substitutions can generally be made in a variety of ways to provide other embodiments of variants within the present invention. In a preferred embodiment, conservative amino acid substitutions are made, as described *supra*.

Within certain aspects, polypeptides, polynucleotides, T cells and/or binding agents described herein may be incorporated into pharmaceutical compositions or immunogenic compositions (*i.e.*, vaccines). Pharmaceutical compositions comprise one or more such compounds and a physiologically acceptable carrier. Vaccines may comprise one or more such compounds and a non-specific immune response enhancer. A non-specific immune response enhancer may be any substance that enhances an immune response to an exogenous antigen. Examples of non-specific immune response enhancers include adjuvants, biodegradable microspheres (*e.g.*, polylactic galactide) and liposomes (into which the compound is incorporated; *see, e.g.*, U.S. Patent No. 4,235,877). Vaccine preparation is generally described in, for example, Powell and Newman, eds., *Vaccine Design* (the subunit and adjuvant approach), Plenum Press (NY, 1995). Vaccines may be designed to generate antibody immunity and/or cellular immunity such as that arising from CTL or CD4+ T cells.

Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other tumor antigens may be present, either incorporated into a fusion polypeptide or as a separate compound, within the composition or vaccine. Polypeptides may, but need not, be conjugated to other macromolecules as described, for example, within US Patent Nos. 4,372,945 and 4,474,757. Pharmaceutical compositions and vaccines may generally be used for prophylactic and therapeutic purposes.

A pharmaceutical composition or vaccine may contain a polynucleotide encoding one or more of the Her-2/neu fusion proteins, *e.g.*, HER-2/neu ECD-ICD and/or HER-2/neu ECD-PD, as described above, such that the fusion protein is generated *in situ*. Such a polynucleotide may comprise DNA, RNA, a modified nucleic acid or a DNA/RNA hybrid. As noted above, a polynucleotide may be present within any of a

variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland (1998) *Crit. Rev. Therap. Drug Carrier Systems* 15:143-198, and references cited therein. Appropriate

5 nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus Calmette-Guerin*) that expresses an immunogenic portion of the fusion protein on its cell surface or secretes such an epitope. In a preferred embodiment, the DNA may be introduced using a viral

10 expression system (*e.g.*, vaccinia, pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:317-321; Flexner *et al.* (1989) *Ann. N.Y. Acad. Sci.* 569:86-103; Flexner *et al.* (1990) *Vaccine* 8:17-21; U.S. Patent Nos. 4,603,112, 4,769,330, 4,777,127 and

15 5,017,487; WO 89/01973; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner (1988) *Biotechniques* 6:616-627; Rosenfeld *et al.* (1991) *Science* 252:431-434; Kolls *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:215-219; Kass-Eisler *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:11498-11502; Guzman *et al.* (1993) *Circulation* 88:2838-2848; and Guzman *et al.* (1993) *Cir. Res.* 73:1202-1207. Techniques for incorporating DNA into such

20 expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer *et al.* (1993) *Science* 259:1745-1749 and reviewed by Cohen (1993) *Science* 259:1691-1692. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells. It will be apparent that a vaccine may comprise both a

25 polynucleotide and a polypeptide component. Such vaccines may provide for an enhanced immune response.

It will be apparent that a vaccine may contain pharmaceutically acceptable salts of the polynucleotides and fusion polypeptides provided herein. Such salts may be prepared from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.*,

30 salts of primary, secondary and tertiary amines and basic amino acids) and inorganic bases (*e.g.*, sodium, potassium, lithium, ammonium, calcium and magnesium salts).

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention

may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer.

- 5 For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for
10 example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,8-2/neu fusion protein may be encapsulated within the biodegradable microsphere or associated with the surface of the microsphere. In one embodiment, an ECD-ICD fusion protein described herein is encapsulated within a biodegradable microsphere. Alternatively or in addition, an ECD-PD fusion protein described herein is encapsulated
15 within a biodegradable microsphere. The microsphere can comprise, *e.g.*, both an ECD-ICD fusion protein and an ECD-PD fusion protein. Preferably the microsphere be less than about 25 μm , preferably about 1 μm to about 10 μm . Encapsulation in liposomes is described, *e.g.*, in U.S. Patent No. 4,235,877.

- Such compositions may also comprise buffers (*e.g.*, neutral buffered saline
20 or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives.
25 Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

- Any of a variety of immune response enhancers or immunostimulatory substances may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen
30 from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck

Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

Within the vaccines provided herein, the adjuvant composition is preferably designed to induce an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.*, IFN- γ , TNF- α , IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman (1989) *Ann. Rev. Immunol.* 7:145-173.

Preferred adjuvants for use in eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL), together with an aluminum salt. MPL adjuvants are available from Corixa Corporation (Hamilton, MT; *see* US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555 and WP 99/33488. Immunostimulatory DNA sequences are also described, for example, by Sato *et al.* (1996) *Science* 273:352. Another preferred adjuvant is a saponin, preferably QS21 (Aquila, United States), which may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent

adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210. QS-21 and 3D-MPL are also described in EP 671 948 B1.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France),
5 SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.*, SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Corixa Corporation, Hamilton, MT), RC-529 (Corixa, USA) and Aminoalkyl glucosaminide 4-phosphates (AGPs).

In a preferred embodiment, the adjuvant is SBAS-2 (*See, e.g.*, EP
10 735898B1).

Any vaccine provided herein may be prepared using well known methods that result in a combination of antigen, immune response enhancer and a suitable carrier or excipient. The compositions described herein may be administered as part of a sustained release formulation (*i.e.*, a formulation such as a capsule or sponge that effects a
15 slow release of compound following administration). Such formulations may generally be prepared using well known technology (*see, e.g.*, Coombes *et al.* (1996) *Vaccine* 14:1429-1438) and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix
20 and/or contained within a reservoir surrounded by a rate controlling membrane.

Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. Such carriers include microparticles of poly(lactide-co-glycolide), as well as polyacrylate, latex, starch, cellulose and dextran. Other delayed-release carriers
25 include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.*, a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see e.g.*, U.S. Patent No. 5, 151, 254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends
30 upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Any of a variety of delivery vehicles may be employed within pharmaceutical compositions and vaccines to facilitate production of an antigen-specific immune response that targets tumor cells. Delivery vehicles include antigen presenting

cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau *et al.* (1998) *Nature* 392:245-251) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (*see* Timmerman *et al.* (1999) *Ann. Rev. Med.* 50:507-529). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel *et al.* (1998) *Nature Med.* 4:594-600).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNF α to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNF α , CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as “immature” and “mature” cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible

intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of Fcγ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.*, CD54 and CD11) and costimulatory molecules (*e.g.*, CD40, CD80, CD86 and 4-1BB).

APCs may generally be transfected with a polynucleotide encoding a fusion protein of the invention (or variant thereof) such that the fusion protein, or a variant thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a composition or vaccine comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi *et al.* (1997) *Immunology and cell Biology* 75:456-460. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the fusion protein of interest, DNA (naked or within a plasmid vector) or RNA; or with fusion protein-expressing recombinant bacterium or viruses (*e.g.*, vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the fusion protein of interest may be covalently conjugated to an immunological partner that provides T cell help (*e.g.*, a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the fusion protein.

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

It will be evident to one skilled in the art that a HER-2/neu fusion protein or nucleic acid for a vaccine may be prepared synthetically or may be naturally derived.

IMMUNE RESPONSE TO FUSION PROTEINS OF THE INVENTION

A. Detection of an immune response to fusion proteins of the invention

In one aspect of the invention, HER-2/neu fusion proteins (or
5 polynucleotides that encode Her-2/neu fusion proteins) are used to generate an immune response to the HER-2/neu protein, including that expressed on a malignancy in which a HER-2/neu oncogene is associated. Representative examples of such malignancies include breast, ovarian, colon, lung and prostate cancers. An immune response to the HER-2/neu protein, once generated by HER-2/neu fusion proteins, can be long-lived and
10 can be detected long after immunization, regardless of whether the protein is present or absent in the body at the time of testing. An immune response to the HER-2/neu protein generated by reaction to a HER-2/neu fusion protein can be detected by examining for the presence or absence, or enhancement, of specific activation of CD4⁺ or CD8⁺ T cells or by antibodies. For example, T cells isolated from an immunized individual by routine
15 techniques (e.g., by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes) are incubated with a HER-2/neu fusion protein. For example, T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with a HER-2/neu fusion protein (typically, 5 µg/ml of whole protein or graded numbers of cells synthesizing HER-2/neu protein). It may be desirable to incubate another aliquot of a T cell sample in
20 the absence of HER-2/neu fusion protein to serve as a control.

Specific activation of CD4⁺ or CD8⁺ T cells may be detected in a variety of ways. Methods for detecting specific T cell activation include detecting the proliferation of T cells, the production of cytokines (e.g., lymphokines), or the generation of cytolytic activity (i.e., generation of cytotoxic T cells specific for a HER-2/neu fusion
25 protein). For CD4⁺ T cells, a preferred method for detecting specific T cell activation is the detection of the proliferation of T cells. For CD8⁺ T cells, a preferred method for detecting specific T cell activation is the detection of the generation of cytolytic activity.

Detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring the
30 rate of DNA synthesis. T cells which have been stimulated to proliferate exhibit an increased rate of DNA synthesis. A typical way to measure the rate of DNA synthesis is, for example, by pulse-labeling cultures of T cells with tritiated thymidine, a nucleoside precursor which is incorporated into newly synthesized DNA. The amount of tritiated

thymidine incorporated can be determined using a liquid scintillation spectrophotometer. Other ways to detect T cell proliferation include measuring increases in interleukin-2 (IL-2) production, Ca^{2+} flux, or dye uptake, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. Alternatively, synthesis of lymphokines (*e.g.*, interferon-gamma) can be measured or the relative number of T cells that can respond to intact p185^{HER-2/neu} protein may be quantified.

B. Detection of antibody production in response to fusion proteins of the invention

10 The present invention is also directed to HER-2/neu fusion proteins that, in addition to being immunogenic to T cells, appear to stimulate B-cells to produce antibodies capable of recognizing HER-2/neu fusion proteins. Detection of such antibodies provides another way to diagnose a malignancy in which a HER-2/neu oncogene is associated with the malignancy. Antibodies specific (*i.e.*, which exhibit a
15 binding affinity of about 10^7 liters/mole or better) for HER-2/neu fusion proteins may be found in a variety of body fluids including sera and ascites. Briefly, a body fluid sample is isolated from a warm-blooded animal, such as a human, for whom it is desired to determine whether antibodies specific for the fusion proteins are present. The body fluid is incubated with HER-2/neu fusion proteins under conditions and for a time sufficient to
20 permit immunocomplexes to form between the Her-2/neu fusion proteins and antibodies specific for the fusion proteins. For example, a body fluid and HER-2/neu fusion proteins may be incubated at 46°C for 24-48 hours. Following the incubation, the reaction mixture is tested for the presence of immunocomplexes. Detection of one or more immunocomplexes formed between HER-2/neu fusion protein and antibodies specific for
25 HER-2/neu fusion protein may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme linked immunosorbent assays (ELISA).

 Suitable immunoassays include the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds.,
30 *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.* (1980) *J. Biol. Chem.*, 255:4980-4983); enzyme-linked immunosorbent assays as described, for example, by Raines *et al.* (1982) *J. Biol. Chem.*, 257:5154-5160; immunocytochemical techniques, including the use of fluorochromes

(Brooks *et al.* (1980) *Clin. Exp. Immunol.*, 39:477); and neutralization of activity (Bowen-Pope *et al.* (1984) *Proc. Natl. Acad. Sci. USA*, 81:2396-2400), all of which are hereby incorporated by reference herein. In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S.

5 Patent Nos.: 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876, all of which are incorporated by reference herein.

For detection purposes, HER-2/neu fusion proteins (*i.e.*, antigens) may either be labeled or unlabeled. When unlabeled, fusion proteins find use in agglutination assays. In addition, unlabeled fusion proteins can be used in combination with labeled
10 molecules that are reactive with immunocomplexes, or in combination with labeled antibodies (second antibodies) that are reactive with the antibody directed against HER-2/neu fusion protein, such as antibodies specific for immunoglobulin. Alternatively, the fusion protein can be directly labeled. Where it is labeled, the reporter group can include, *e.g.*, radioisotopes, fluorophores, enzymes, luminescers, dye particles and the like. These
15 and other labels are well known in the art and are described, for example, in U.S. Patent Nos.: 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402, all of which are incorporated by reference herein.

Typically in an ELISA assay, the fusion protein of interest is adsorbed to the surface of a microtiter well. Residual protein-binding sites on the surface are then
20 blocked with an appropriate agent, such as bovine serum albumin (BSA), heat-inactivated normal goat serum (NGS), or BLOTTO (buffered solution of nonfat dry milk which also contains a preservative, salts, and an antifoaming agent). The well is then incubated with a sample suspected of containing specific antibody. The sample can be applied neat, or, more often, it can be diluted, usually in a buffered solution which contains a small
25 amount (0.1% -5.0% by weight) of protein, such as BSA, NGS, or BLOTTO. After incubating for a sufficient length of time to allow specific binding to occur, the well is washed to remove unbound protein and then incubated with an anti-species specific immunoglobulin antibody labeled with a reporter group. The reporter group can be chosen from a variety of enzymes, including, *e.g.*, horseradish peroxidase, beta-
30 galactosidase, alkaline phosphatase, and glucose oxidase. Sufficient time is allowed for specific binding to occur, then the well is again washed to remove unbound conjugate, and the substrate for the enzyme is added. Color is allowed to develop and the optical density of the contents of the well is determined visually or instrumentally.

In one preferred embodiment of this aspect of the present invention, a reporter group is bound to the HER-2/neu fusion protein of interest. The step of detecting immunocomplexes involves removing substantially any unbound HER-2/neu fusion protein and then detecting the presence or absence of the reporter group.

5 In another preferred embodiment, a reporter group is bound to a second antibody capable of binding to the antibodies specific for HER-2/neu fusion proteins. The step of detecting immunocomplexes involves (a) removing substantially any unbound antibody, (b) adding the second antibody, (c) removing substantially any unbound second antibody and then (d) detecting the presence or absence of the reporter group. Where the
10 antibody specific for HER-2/neu fusion proteins is derived from a human, the second antibody is an anti-human antibody.

In a third preferred embodiment for detecting immunocomplexes, a reporter group is bound to a molecule capable of binding to the immunocomplexes. The step of detecting involves (a) adding the molecule, (b) removing substantially any
15 unbound molecule, and then (c) detecting the presence or absence of the reporter group. An example of a molecule capable of binding to the immunocomplexes is protein A.

It will be evident to one skilled in the art that a variety of methods for detecting the immunocomplexes may be used within the present invention. Reporter groups suitable for use in any of the methods include, *e.g.*, radioisotopes, fluorophores,
20 enzymes, luminescers, and dye particles.

In a related aspect of the present invention, detection of immunocomplexes formed between HER-2/neu fusion proteins and antibodies in body fluid which are specific for HER-2/neu fusion proteins may be used to monitor the effectiveness of cancer therapy, which involves a HER-2/neu fusion protein, for a malignancy in which
25 the HER-2/neu oncogene is associated. Samples of body fluid taken from an individual prior to and subsequent to initiation of therapy may be analyzed for the immunocomplexes by the methodologies described above. Briefly, the number of immunocomplexes detected in both samples are compared. A substantial change in the number of immunocomplexes in the second sample (post-therapy initiation) relative to
30 the first sample (pre-therapy) reflects successful therapy.

CANCER THERAPY

In further aspects of the present invention, the compositions described herein may be used for immunotherapy of cancer, such as breast, ovarian, colon, lung and

prostate cancer. Within such methods, pharmaceutical compositions and vaccines are typically administered to a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may or may not be afflicted with cancer. Accordingly, the above pharmaceutical compositions and vaccines may be used to

5 prevent the development of a cancer or to treat a patient afflicted with a cancer. A cancer may be diagnosed using criteria generally accepted in the art, including the presence of a malignant tumor. Pharmaceutical compositions and vaccines may be administered either prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs. Administration

10 may be by any suitable method, including administration by intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, intradermal, anal, vaginal, topical, sublingual and oral routes.

Within certain embodiments, immunotherapy may be active immunotherapy, in which treatment relies on the *in vivo* stimulation of the endogenous

15 host immune system to react against tumors with the administration of immune response-modifying agents (such as fusion polypeptides and polynucleotides as provided herein).

Within other embodiments, immunotherapy may be passive immunotherapy, in which treatment involves the delivery of agents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or

20 indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T cells as discussed *supra*, T lymphocytes (such as CD8⁺ cytotoxic T lymphocytes and CD4⁺ T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and antigen-presenting cells (such as dendritic cells and

25 macrophages) expressing a fusion protein provided herein. T cell receptors and antibody receptors specific for the fusion polypeptides recited herein may be cloned, expressed and transferred into other vectors or effector cells for adoptive immunotherapy. The fusion polypeptides provided herein may also be used to generate antibodies or anti-idiotypic antibodies (as described above and in U.S. Patent No. 4,918,164) for passive

30 immunotherapy.

Effector cells may generally be obtained in sufficient quantities for adoptive immunotherapy by growth *in vitro*, as described herein. Culture conditions for expanding single antigen-specific effector cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. Such *in vitro* culture conditions

typically use intermittent stimulation with antigen, often in the presence of cytokines (such as IL-2) and non-dividing feeder cells. As noted above, immunoreactive fusion polypeptides as provided herein may be used to rapidly expand antigen-specific T cell cultures in order to generate a sufficient number of cells for immunotherapy. In

5 particular, antigen-presenting cells, such as dendritic, macrophage or B cells, may be pulsed with immunoreactive fusion polypeptides or transfected with one or more polynucleotides using standard techniques well known in the art. For example, antigen-presenting cells can be transfected with a polynucleotide having a promoter appropriate for increasing expression in a recombinant virus or other expression system. Cultured
10 effector cells for use in therapy must be able to grow and distribute widely, and to survive long term *in vivo*. Studies have shown that cultured effector cells can be induced to grow *in vivo* and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (*see*, for example, Cheever *et al.* (1997) *Immunological Reviews* 157:177).

15 Alternatively, a vector expressing a fusion polypeptide recited herein may be introduced into antigen presenting cells taken from a patient and clonally propagated *ex vivo* for transplant back into the same patient. Transfected cells may be reintroduced into the patient using any means known in the art, preferably in sterile form by intravenous, intracavitary, intraperitoneal or intratumor administration.

20 Routes and frequency of administration of the therapeutic compositions described herein, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally.
25 Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response, and is at
30 least 10-50% above the basal (*i.e.*, untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial or longer disease-free

survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more fusion polypeptides, the amount of each fusion protein present in a dose ranges from about 1 µg to 5 mg, preferably 100 µg to 5 mg, and most preferably 5 µg to 250 µg per kg of host. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 ml to about 5 ml.

Preferably, an initial or primary immunization will be made with a Her-2/neu fusion protein having, e.g., at least one of an ECD and/or a ICD or PD, and a subsequent or booster immunization will be made with a Her-2/neu fusion protein having, e.g., at least one of a ECD and/or a ICD or PD. Preferred ECD-ICD and/or ECD-PD fusion proteins for immunization include those described herein. It will be appreciated by one skilled in the art that the present invention contemplates the use of an intact HER-2/neu fusion protein as well as division of the Her-2/neu fusion protein into a plurality of peptides. Neither intact p185^{HER-2/neu} protein nor a peptide having the amino acid sequence of the entire HER-2/neu ECD domain (or a portion of the HER-2/neu ECD domain) are used alone for immunization.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (e.g., more frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to a HER-2/neu protein or fusion protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

DETECTING CANCER

A. Methods of detecting cancer

In general, a cancer may be detected in a patient based on the presence of HER-2/neu proteins and/or polynucleotides encoding such proteins in a biological sample (such as blood, sera, plasma, urine and/or tumor biopsies) obtained from the patient. In other words, such proteins may be used as markers to indicate the presence or absence of a cancer such as, e.g., breast, ovarian, colon, lung, prostate cancer, *etc.* The binding

agents provided herein generally permit detection of the level of HER-2/neu protein that binds to the agent in the biological sample. Polynucleotide primers and probes may be used to detect the level of mRNA encoding a HER-2/neu tumor protein, which is also indicative of the presence or absence of a cancer. In general, a HER-2/neu tumor
5 sequence should be present at a level that is at least three fold higher in tumor tissue than in normal tissue

There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. *See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In
10 general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined cut-off value.

In a preferred embodiment, the assay involves the use of binding agent
15 immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to the polypeptide or an antibody or other agent that specifically binds
20 to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity
25 of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length HER-2/neu tumor proteins and portions thereof to which the binding agent binds, and HER-2/neu fusion proteins and portions thereof to which the binding agent binds, as described above.

The solid support may be any material known to those of ordinary skill in
30 the art to which the tumor protein may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S.

Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μ g, and preferably about 100 ng to about 1 μ g, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.,* Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody.

Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20TM (Sigma Chemical Co., St. Louis, MO). The immobilized

antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with breast, ovarian, colon, lung or prostate cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time.

At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer, such as breast, ovarian, colon, lung or prostate cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value for the detection of a cancer is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without the cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value

is considered positive for the cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett *et al.*, *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for a cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1 μ g, and more preferably from about 50 ng to about 500 ng. Such

tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the tumor proteins, or binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use HER-2/neu polypeptides to detect antibodies that bind to such polypeptides in a biological sample. The detection of such HER-2/neu protein specific antibodies may correlate with the presence of a cancer.

A cancer may also, or alternatively, be detected based on the presence of T cells that specifically react with a HER-2/neu fusion protein in a biological sample. Within certain methods, a biological sample comprising CD4⁺ and/or CD8⁺ T cells isolated from a patient is incubated with a HER-2/neu fusion polypeptide, a polynucleotide encoding such a fusion polypeptide and/or an APC that expresses at least such fusion polypeptide, and the presence or absence of specific activation of the T cells is detected. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with a HER-2/neu fusion polypeptide (*e.g.*, 5 - 25 µg/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of HER-2/neu fusion polypeptide to serve as a control. For CD4⁺ T cells, activation is preferably detected by evaluating proliferation of the T cells. For CD8⁺ T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a cancer in the patient.

As noted above, a cancer may also, or alternatively, be detected based on the level of mRNA encoding a HER-2/neu protein in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a HER-2/neu cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (*i.e.*, hybridizes to) a polynucleotide encoding the HER-2/neu protein. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide probes that specifically hybridize to a

polynucleotide encoding a HER-2/neu protein or fusion protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the HER-2/neu protein in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a HER-2/neu protein, or fusion protein, that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Preferably, oligonucleotide primers and/or probes hybridize to a polynucleotide encoding a HER-2/neu protein or fusion protein described herein under moderately stringent conditions, as defined above. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. In a preferred embodiment, the oligonucleotide primers comprise at least 10 contiguous nucleotides, more preferably at least 15 contiguous nucleotides, of a DNA molecule having a sequence recited SEQ ID NOS:6 or 7. Techniques for both PCR based assays and hybridization assays are well known in the art (*see, for example, Mullis et al. (1987) Cold Spring Harbor Symp. Quant. Biol., 51:263; Erlich ed., PCR Technology, Stockton Press, NY, 1989*).

One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a biological sample such as a biopsy tissue and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on biological samples taken from a test patient and from an individual who is not afflicted with a cancer. The amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

In another embodiment, HER-2/neu proteins or fusion proteins and polynucleotides encoding such proteins or fusion proteins may be used as markers for monitoring the progression of cancer. In this embodiment, assays as described above for the diagnosis of a cancer may be performed over time, and the change in the level of reactive polypeptide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In

general, a cancer is progressing in those patients in whom the level of polypeptide detected by the binding agent increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide either remains constant or decreases with time.

5 Certain *in vivo* diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

10 As noted above, to improve sensitivity, multiple HER-2/neu fusion protein markers may be assayed within a given sample. It will be apparent that binding agents specific for different proteins provided herein may be combined within a single assay. Further, multiple primers or probes may be used concurrently. The selection of tumor protein markers may be based on routine experiments to determine combinations that
15 results in optimal sensitivity. In addition, or alternatively, assays for tumor proteins provided herein may be combined with assays for other known tumor antigens.

B. Diagnostic kits

 The present invention further provides kits for use within any of the above
20 diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a HER-2/neu fusion protein. Such antibodies or fragments may be provided attached to a support material, as described
25 above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

 Alternatively, a kit may be designed to detect the level of mRNA encoding
30 a HER-2/neu protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a HER-2/neu protein or fusion protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or

container to facilitate the detection of a polynucleotide encoding a HER-2/neu protein.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application
5 were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit
10 or scope of the appended claims.

EXAMPLES

The following examples are offered by way of illustration, and are not intended to limit the scope of the present invention or appended claims.

15 In the examples, general molecular biology reagents, such as oligonucleotide primers, lipofectamine and restriction endonucleases, were primarily obtained from Gibco/BRL (Grand Island, NY). Restriction endonucleases, Aat II and PflM-1 were obtained from New England Biolabs (Beverly, MA). HER-2/neu ELISA assay kit and HER-2/neu specific monoclonal antibody Ab-3 were purchased from
20 Oncogene Science (Manhasset, NY). The pFLAGCMV-1 expression vector and FLAG-Tag M2 Antibody were purchased from Kodak (Rochester, NY). Pfu Polymerase was obtained from Stratagene (La Jolla, CA). The pcDNA3.1/hyg expression vector was purchased from Invitrogen (Carlsbad, CA)

Example 1

CLONING OF THE ICD, KD AND PD FRAGMENTS OF HER-2/NEU INTO THE pFLAGCMV-1 EXPRESSION VECTOR

25 The DNA fragments of HER-2/neu that encode the intracellular domain (ICD), kinase domain (KD) and phosphorylation domain (PD) were obtained separately
30 by polymerase chain reaction. Restriction digestion sites, Hind III and Xho I, were introduced at their 5' and 3' end respectively. This design allowed the cloning the DNA fragments into the pFLAGCMV-1 expression vector (Kodak) in frame with a preprotrypsin leader sequence and a FLAG Tag sequence at their N-terminus. The PCR products were gel purified and cloned into the Hind III and Sal I sites of pFLAGCMV-1.

The resulting expression plasmids were designated as pFLAGCMV-1/ICD (Fig. 1), pFLAGCMV-1/KD (Fig. 2) and pFLAGCMV-1/PD (Fig. 3).

Example 2

CLONING OF THE ECD-PD FUSION PROTEIN INTO THE pcDNA3.1/hyg EXPRESSION VECTOR

The DNA fragment encoding the HER-2/neu PD was amplified by polymerase chain reaction. After gel purification, it was cloned into the Aat II and Xho I sites of the pT7-HER-2/neu plasmid. This procedure generated a new cloning vector, pT7/ECD-PD which linked the ECD and PD together (including a Ser from the transmembrane domain). The pT7/ECD-PD plasmid was digested with Hind III and Xho I at 37°C for 1 hour. The 2.7 kb DNA fragment encoding the ECD-PD fusion protein was gel purified and subcloned into the Hind III and Xho I sites of pcDNA3.1/hyg (Invitrogen). The resulting expression vector was designated as pcDNA3.1/hyg/ECD-PD (Fig. 5).

Example 3

EXPRESSION OF THE ICD, KD AND PD FRAGMENTS OF HER-2/NEU IN HEK-293 CELLS

The pFLAGCMV-1 expression plasmid (Kodak) was used to determine which region of the HER-2/neu intracellular domain could be secreted in culture media. The proteins were expressed as fusions with a preprotrypsin secretion signal and a FLAG-Tag at their N-terminus, as described in Example 1.

Transfection and growth of ICD, KD and PD expressing cell lines was conducted as follows: human embryonic kidney fibroblasts (HEK-293 cells) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. One day prior to transfection, 1.5×10^5 cells were seeded into each well of a six-well dish. Transfection was carried out using 1 µg plasmid DNA by lipofectamine (Gibco/BRL) in serum free media. Culture media and cells were harvested 72 hours later. For selection of stable transformants, the transfected cells were grown in media containing 200 µg/ml hygromycin.

Cells and culture media were assayed for FLAG-Tag fusion proteins by Western blot analysis as follows: culture media and cell lysate from transfected HEK-293

cells were separated on 7.5% SDS polyacrylamide gel. The proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) filters. The PVDF filters were first incubated with 5% bovine serum albumin in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20), then incubated for 1 hour with the primary antibody, and finally
5 incubated for another hour with peroxidase-conjugated goat anti-mouse antibody. Immunoblots were developed using ECL system (Amersham Corp.). The mouse monoclonal antibody, c-neu Ab-3 ("Ab-3") (Oncogene Science), was used for detecting the HER-2/neu protein. Ab-3 recognizes the carboxyl terminal of the human HER-2/neu protein. For detecting FLAG-Tag fusion proteins, the M2 monoclonal antibody (Kodak)
10 was used as primary antibody in the analysis.

The results presented in Fig. 4 show that neither full length ICD nor KD was secreted, but that PD was detected in the culture media. The results indicate that the structure of KD did not permit passage of the protein through the cell membrane.

Example 4

EXPRESSION OF THE ECD-PD FUSION PROTEIN IN HEK-293 AND CHO CELLS USING THE pcDNA3.1/hyg EXPRESSION VECTOR

The ECD-PD fusion protein with a preprotrypsin secretion signal and FLAG-Tag at its N-terminus was constructed, as described in Example 2.

20 Transfection and growth of ECD-PD expressing cell lines was conducted as follows: HEK-293 cells and Chinese hamster ovary cells (CHO) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. One day prior to transfection, 1.5×10^5 cells were seeded into each well of a six-well dish. Transfection was carried out using 1 μ g of plasmid DNA by lipofectamine (Gibco/BRL) in serum free
25 media. Culture media and cells were harvested 72 hour later. For selection of stable transformants, the transfected cells were grown in media containing 200 μ g/ml hygromycin.

The secretion of the soluble ECD-PD fusion protein was determined by an ELISA assay with HER-2/neu ECD specific antibodies as follows: the microplate was
30 pre-coated with the HER-2/neu specific mouse antibody (Oncogene Science) to capture HER-2/neu protein in the samples. Test samples were incubated in the microplate overnight at room temperature, incubated for 1 hour with detector antibody, and incubated another hour with a horseradish peroxidase conjugated goat anti-rabbit

antibody. After adding peroxidase substrate o-phenylenediamine, a colored product was formed. The colored product was quantitated by spectrophotometry. Absorbance at 490 nm reflected the amount of neu protein in the samples.

Thereafter, the secretion of the soluble ECD-PD fusion protein was determined by Western blot analysis with HER-2/neu PD specific antibodies as follows: culture media and cell lysate from transfected HEK-293 cells were separated on 7.5% SDS polyacrylamide gel. For western blot analysis, the proteins were transferred electrophoretically to PVDF filters. The PVDF filters were incubated with 5% bovine serum albumin in TBST, incubated for 1 hour with the primary antibody, and incubated for another hour with peroxidase-conjugated goat anti-mouse antibody. Immunoblots were developed using the ECL system (Amersham Corp.). The mouse Ab-3 monoclonal antibody (Oncogene Science), was used for detecting the HER-2/neu protein in the present experiments. Ab-3 recognizes the carboxyl terminal of human HER-2/neu protein. For detecting FLAG-Tag fusion proteins, M2 monoclonal antibody (Kodak) was used as primary antibody in the analysis. The results are presented in Fig. 6.

Example 5

CLONING OF THE HUMAN ECD-APD FUSION PROTEIN INTO THE pcDNA3.1/hyg EXPRESSION VECTOR

The human ECD-APD fusion protein, shown in Fig. 13 (SEQ ID NO:7), was prepared by polymerase chain reaction (PCR) with the following primers:

PDM-251 5'-cctgaatcgcggaacccaagtgtgcaccggcac-3' (SEQ ID NO:15) Tm 69°C
PDM-279 5'-ctggactcgagtcattagcgggtgctgtggtgg-3' (SEQ ID NO:16) Tm 69°C.

The polymerase chain reaction conditions were: 10 µl 10x Pfu Buffer (Stratagene), 1 µl 10 mM dNTPs, 2 µl 10 µM each oligo, 83 µl sterile water, 1.5 µl Pfu DNA polymerase, and 50 ng template at 96°C for 2 minutes x 1 cycle; (96°C for 20 seconds, 69°C for 15 seconds, 72°C for 5 minutes) x 40 cycles; 72°C for 5 minutes x 1 cycle. The PCR product was digested with Nru I and Xho I, and cloned into a pPDM His vector (a modified pET28 vector which has a His tag-in-frame with a blunt Restriction enzyme cutter Eco 72I), which was cut with Eco 72I and Xho I. The sequence was confirmed and the recombinant plasmid was then transformed into BL21 pLys S for *E. coli* expression. The plasmid construct was then digested with BamHI and XhoI and cloned into pcDNA3.1/hyg/ECD-PD which was cut with the same restriction enzymes.

Example 6

EXPRESSION IN *E. coli* of HUMAN ECD-PD-C_T-HIS TAG FUSION PROTEIN

The human ECD-PD fusion protein was cloned into the pcDNA3.1/hyg
5 vector as described in example 2 and was used as template for constructing the hECD-PD
in frame with a C-terminal 6 x histidine tag. The hECD-PD was amplified by PCR using
the following primers:

AWO28 hECD-PD sense primer, with Nco I site:

5'-GGGccatgggAGCACCCAAGTGTGCACCGGC-3 (SEQ ID NO:17)

10 AWO29 hECD-PD antisense primer, with Xho I site without stop:

5'-GGGctcgagCACTGGCACGTCCAGACCCAGG-3' (SEQ ID NO:18)

The PCR product was then cut with the Nco I and Xho I restriction
enzymes, purified, and ligated into the pET28b expression vector linearized with the same
two restriction enzymes. The ligation product was transformed into NovaBlue cells and
15 several colonies were selected for screening. Of those, the hECD-PD.C_This clones were
confirmed by DNA sequence and used for subsequent protein expression.

For protein expression, a hECD-PD.C_This clone was transformed into
BL21 (DE3) CodonPlus-RIU *E. coli* competent cells. A standard mini expression screen
was carried out with clones from the transformation to determine the induction yield. The
20 best results were obtained when the cells were grown in TB media at 37°C for 2 hours.

The *E. coli* produced hECD-PD.C_This was then purified on a monoQ
column, and refolded in 20 mM Tris-HCL (pH 8.0) buffer. The refolded protein was
tested and found to be positive for Herceptin binding by Western blot and ELISA (Fig.
17). The Herceptin binding activity was, however, lost later on, probably due to
25 denaturation of the protein.

Example 7

EXPRESSION IN *E. coli* of HUMAN N_T-HIS TAG-ECD-PD FUSION PROTEIN

The human ECD-PD fusion protein was cloned into the pPDM expression
30 vector with a 5'Nde I and a 3' Xho I restriction sites. The ECD-PD insert was fused in
frame with a N-terminal 6 x histidine tag.

For protein expression, the pPDM vector containing the N_This-hECD-PD
fusion protein was transformed into BL21 (DE3) CodonPlus-RIU *E. coli* competent cells.
A standard mini expression screen was carried out with clones from the transformation to

determine the induction yield. The best results were obtained when the cells were grown in TB media at 30°C for 2 hours.

The *E. coli* derived unpurified N_This-hECD-PD fusion protein was recognized by the mouse c-neu-3 antibody and by a rabbit anti-ECD antibody. Following
5 purification, the *E. coli* derived N_This-hECD-PD was recognized by Herceptin both in Western blots and in ELISA assays (Fig. 17).

Example 8

EXPRESSION IN *E. coli* of MOUSE ECD-PD-C_T HIS TAG FUSION PROTEIN

10 The mouse ECD-PD fusion protein was cloned into the pcDNA3.1 vector following a protocol similar to that described in example 2 for cloning the human ECD-PD fusion protein. This construct was then used as template for constructing the mECD-PD in frame with a C-terminal 6 x histidine tag followed by a stop codon. The internal
15 Nco I site in the mECD-PD/pcDNA3.1 construct (base pair 1932 of the ORF) was silently mutated by site-directed mutagenesis using the following primers:

AW038 primer:

5'-GGCCCCTCCAGCCCCGATGGACAGCACCTTCTACCG-3' (SEQ
NO:19)

AW039 primer:

20 5'-CGGTAGAAGGTGCTGTCCATCGGGGCTGGAGGGGCC-3' (SEQ
ID NO:20)

After the sequence was confirmed, the mECD-PD fusion construct was amplified by PCR using the following primers:

AW036 sense primer, with Nco I restriction site:

25 5'-GGGccatggGTACCCAAGTGTGTACCGG-3' (SEQ ID NO:21)

AW037 antisense primer, with Xho I restriction site:

5'-GGGctcgagTCAATGGTGATGGTGATGGTGTCATGG
CACATCCAGGCCTAGGTACTCAGGG-3' (SEQ ID NO:22)

The PCR product was then cut with the Nco I and Xho I restriction
30 enzymes, purified, and ligated into the pET28b expression vector linearized with the same two restriction enzymes.

The ligation product was transformed into NovaBlue and yielded multiple colonies. Four colonies were selected for sequence analysis. Of those, a mECD-PD.C_This clone which had the correct sequence was transformed into BL21 (DE3)

CodonPlus-RIU *E. coli* competent cells. A standard mini expression screen was carried out with clones from the transformation to determine the induction yield. The best results were obtained when the cells were grown in 2xYT media at 30°C for 3 hours.

5

Example 9

EXPRESSION IN *E. coli* of the Ra12-mECD-PD-C_T HIS TAG FUSION PROTEIN

The mouse ECD-PD fusion protein was cloned into the pET28b expression vector as described in Example 8. The Ra12 sequence was amplified using the following PCR fragments that added Nco I sites on the 5' and 3' ends:

10 Ra12.JC05: 5'-CCGccatggGCACGGCCGCGTCCGATAACTTCC-3'
(SEQ ID NO:23)

Ra12.JC06: 5'-GCGccatggCGGCCGGGGTCCCTCGGCC-3' (SEQ
ID NO:24)

To obtain the Ra12 adjuvant fusion with mECD-PD, the Ra12 PCR
15 product was then digested with the Nco I restriction enzyme, and ligated into the Nco I
digested and CIAP treated pET28b-mECD-PD vector. The ligation product was
transformed into NovaBlue cells and yielded multiple colonies. Due to the non-
directional specific ligation reaction, twice as many clones were picked for plasmid
miniprep. These clones were screened by digestion with the Afi III restriction enzyme for
20 correct orientation of the insert. The sequence of a few correctly oriented clones was
analyzed. One clone with a correct sequence was transformed into BL21 (DE3)
CodonPlus-RIU *E. coli* competent cells for expression. A standard mini expression
screen was carried out with clones from the transformation to determine the induction
yield. The best results were obtained when the cells were grown in LB media at 37°C for
25 3 hours.

Example 10

EXPRESSION IN *E. coli* of LeIF.mECD-PD-C_T HIS TAG FUSION PROTEIN

The mouse ECD-PD fusion protein was cloned into the pET28b expression
30 vector as described in Example 8. The LeIF sequence was amplified using the following
PCR fragments that added Nco I sites on the 5' and 3' ends:

LeIF.JC03: 5'-CGCccatggCGCAGAATGATAAGATCGCCC-3' (SEQ
ID NO:25)

LeIF.JC04: 5'-GCCccatggCGTCGCGCATGAACTTCTTCGTC-3'
(SEQ ID NO:26)

To obtain the LeIF adjuvant fusion with mECD-PD, the LeIF PCR product was then digested by the Nco I restriction enzyme, and ligated into the Nco I digested and
5 CIAP treated pET28b-mECD-PD vector. The ligation product was transformed into NovaBlue cells and yielded multiple colonies. Due to the non-directional specific ligation reaction, twice as many clones were picked for plasmid miniprep. These clones were screened by digestion with the Kpn I restriction enzyme for correct orientation of the insert. The sequence of a few correctly oriented clones was analyzed. One clone with
10 a correct sequence was transformed into BL21 (DE3) CodonPlus-RIU *E. coli* competent cells for expression. A standard mini expression screen was carried out with clones from the transformation to determine the induction yield. The best results were obtained when the cells were grown in 2xYT media at 30°C for 3 hours.

15

Example 11

EXPRESSION IN PICHIA OF AN ECD-PD FUSION PROTEIN

The ECD-PD recombinant protein used for expression in *Pichia* had the same design as for CHO expression with two modifications: (i) the native secretion signal sequence of the HER-2/neu gene had been replaced by the *Saccharomyces cerevisiae*
20 alpha pre-pro signal sequence; and (ii) the C-terminal part of the recombinant protein was elongated by one glycine and six histidines.

The ECD-PD fusion protein expression cassette was integrated into the SMD1168 *Pichia* strain using the Spheroplast method. Six multicopy integrant clones were selected among 250 clones by quantitative Dot Blot analysis. The selected clones
25 were induced during 72 hours in Buffered Methanol-complex medium (BMMY-1% methanol) in shake-flasks conditions. The six candidate clones showed the same expression profile in the cell-free supernatants and in total cellular extracts.

In the cell-free supernatants, secretion of full-length ECD-PD recombinant protein was very weak and only detected on Western Blots using the c-neu-3 mouse
30 antibody (Calbiochem). Secretion and accumulation (maximum after 72 hours) of a \pm 70kDa protein was visible on Silver stained SDS-PAGE and detected on Western blot under non-reducing conditions with Herceptin mouse antibody. This protein was not detected using the mouse c-neu-3 antibody or a mouse anti-histidine antibody (QIAGEN).

In total cellular extracts, no specific band was detected on SDS-PAGE using the Silver staining DAIICHI coloration kit. Two bands were detected on Western blots using the mouse c-neu-3 or the mouse anti-histidine antibody. One band has the same molecular size as that observed for the secreted ECD-PD product following expression in CHO cells. The other band appeared as a "smear" ranging from 100-120kDa. These two signals could correspond to ECD-PD recombinant proteins retained in the E.R. and presenting various forms of glycosylations.

Example 12

10 EXPRESSION IN CHOK1 CELLS OF ECD-PD AND ECD-APD FUSION PROTEINS

The pcDNA3.1/hyg/ECD-PD and pcDNA3.1/hyg/ECD-APD plasmids were digested by the Xba I restriction enzyme and the DNA fragments encompassing the ECD-PD and ECD-APD fusion proteins were gel purified as Xba I fragments of 2783 and 2166 bp, respectively. Each fragment was transferred into the pEE14-GS vector (CellTech) linearized with Xba I (cloning site downstream of the CMV immediate early promoter). After ligation, transformation was performed into DH5 α competent *E. coli* cells. Out of sixteen colonies analyzed by restriction enzyme digestion, 2 positive colonies were found for ECD-PD, and 1 positive colony was found for ECD-APD. The obtained plasmids were prepared at large scale and purified by double CsCl-EtBr gradient centrifugation. The plasmids were analyzed by restriction enzyme digestion and sequencing of the 5' and 3' junctions between insert and vector and no abnormalities were found.

Transfection of CHO-K1 cells derived from Master Cell Bank MCB CHO-K1 028W 1996/2 SHF P31, growing under suspension serum free conditions, was carried out with both the pEE14-ECD-PD and the pEE14-ECD-APD plasmids using the classical DNA Ca Phosphate co-precipitation technique. Cells were counted 48 hours after transfection and transferred into 96-well plates at a density of 5000 cells/well. Transfected cells were selected according to the procedure of the glutamine synthetase (GS) expression system described by Crockett *et al.* ((1990) *Biotech.*, 8: 662) and amplified in the presence of 30 μ M methionine sulfoximine (MSX) in GMEM medium containing no glutamine and supplemented with additives (glutamate/asparagine/nucleosides) and 5% dialysed Fetal Bovine Serum (FBS). Cells were washed three times during the first week and twice during the second week

following transfection. During the third wash, 20% conditioned medium was added. During the fifth wash, the concentration of MSX was raised to 50 μ M to increase the level of selection.

The MSX transfectant clones were transferred 3-5 weeks after transfection into 24-well plates and the culture supernatants were harvested. Expression of the ECD-PD or ECD- Δ PD fusion proteins was tested by Western blot analysis using Herceptin antibody under non reducing conditions. Expression of the ECD-PD fusion protein was detected in 18 out of 52 clones tested, while 13 out of 47 clones tested were positive for ECD- Δ PD expression. The selected clones expressing the fusion proteins were then readapted to suspension serum-free conditions. Based on the level of expression, growth and viability, 5 clones carrying the ECD-PD construct and 3 clones carrying the ECD- Δ PD construct were further evaluated and characterized. For the ECD-PD construct, clone 560 F3 showed the highest expression level.

Expression was evaluated at 33°C in the presence or absence of sodium butyrate (2 mM) and of DMSO (2 %). Some of the clones were inducible by NaB or DMSO. Expression in CHO-K1 cells of ECD-PD and ECD- Δ PD was analyzed by Western blots and SDS-PAGE followed by either Silver or Coomassie staining. The Herceptin and the c-neu-3 mouse monoclonal antibodies, as well as the 8029K rabbit polyclonal antibody were used for Western blot analysis. Analysis of the culture supernatants from ECD-PD and ECD- Δ PD clones showed a band in Coomassie/Silver stained gels at 150 kDa and at 98 kDa, respectively. The same bands were revealed by Herceptin and by the 8029K polyclonal antiserum, as well as by the c-neu-3 antibody for ECD-PD only (Fig. 18). The CHO-expressed HER-2/neu fusion proteins are recognized by the Herceptin antibody (Fig. 18).

The expression level of the fusion proteins was also followed in terms of stability during the different cell passages. Five ECD-PD clones and two ECD- Δ PD clones were followed during passages and the stability of expression was evaluated by Western blot analysis. Out of the seven analyzed clones, four were stable after more than 32 passages, although one of them showed a high mortality and the other presented big cells.

Small scale production runs were carried out with the two best ECD-PD and ECD- Δ PD clones. Cells were cultured in suspension under serum-free conditions for 120 hours at 33°C in the presence of 2 mM Sodium butyrate. The expression of both

fusion proteins was evaluated by Western blot using the Herceptin antibody and by SDS-PAGE followed by silver staining using the Daiichi kit. Both fusion proteins were found to be expressed at +/- 100 µg/ml.

5

Example 13

PURIFICATION OF ECD-PD AND ECD-ΔPD FUSION PROTEINS FOLLOWING EXPRESSION IN CHO CELLS

Following expression in CHO cells and secretion, the ECD-PD and ECD-ΔPD fusion proteins were purified by anion exchange chromatography on Q sepharose
10 High Performance columns. Before loading the supernatant onto the column, the pH was adjusted to 6.5 by adding 1N HCl. For the chromatography, 1 ml of Q Sepharose High Performance resin (Pharmacia) was used for a C10/10 column (Pharmacia).

The column was first equilibrated with 10 column volumes of H₂O at 4 ml/min, followed by 1 column volume of 0.5 M NaOH at 4 ml/min, and 10 column
15 volumes of Buffer A (20 mM Bis-Tris propane pH 6.5 – 50 mM NaCl) at 4 ml/min. The sample was then loaded onto the column and allowed to pass through at a flow rate of 1 ml/min. The column was then washed with Buffer A at 1 ml/min until the O.D. at 280 nm reached 0.1, and an additional wash step of 20 column volumes was then performed. Before elution, the flow stream was reversed and an additional wash step of 3 column
20 volumes was performed.

The elution was carried out at 1 ml/min, first with Buffer B (20 mM Bis-Tris propane pH 6.5-250 mM NaCl), and then with Buffer C (20 mM Bis-Tris propane pH 6.5-1 mM NaCl). The fusion proteins of interest were eluted with buffer B.

The fusion proteins were further purified by hydrophobic chromatography
25 on Phenyl Sepharose 6 Fast Flow low substitution. The eluate containing the ECD-PD and ECD-ΔPD fusion proteins (Buffer B eluate) was adjusted to obtain a concentration of 1M ammonium sulfate (AMS) by addition of solid AMS (140g/liter of solution). The pH of the solution was checked to be 7.0.

For the chromatography, 0.5 ml of Phenyl Sepharose 6 Fast Flow low
30 substitution (Pharmacia) was used with a C10/10 (Pharmacia). The column was then equilibrated with 10 column volumes of H₂O, at 4 ml/min, 1 column volume of 0.5 M NaOH, at 4 ml/min, and 10 column volumes of buffer D (1 mM PO₄ pH 7.0 – 1M AMS), at 4 ml/min. Following equilibration, the sample was loaded and allowed to pass through

the column at a flow rate of 0.5 ml/min. The column was then washed in Buffer D at 0.5 ml/min until the O.D. at 280nm reached the baseline, and then at 1 ml/min for 10 column volumes. Before elution, the flow stream was reversed and an additional wash step of 3 column volumes was performed. Elution was carried out at 1 ml/min with buffer E (1 mM PO₄ pH 7.0).

The purified fusion proteins were analyzed by SDS-PAGE followed by silver staining using the Daiichi kit, and by Western blot, using the 8029K rabbit polyclonal antibody or the mouse Herceptin antibody. The analysis showed that the level of purity following the two purification steps was estimated at +/- 90% by densitometry (Biorad GS-700 Imaging Densitometer). The Western blot analysis showed that the monomers remained the major band all along the purification, that the level of oxydation was not increased, and that the detection of the epitope of interest was not modified by the conditions of purification, as shown by using the Herceptin antibody. The total amount of each fusion protein recovered was measured using a colorimetric protein assay (DOC TCA BCA). This assays estimated that 2 and 4 mg of ECD-PD and ECD-ΔPD fusion protein, respectively, were purified from 75 ml of culture, with a level of purity of +/- 90%.

WHAT IS CLAIMED IS:

- 1 1. An isolated protein comprising a HER-2/neu extracellular domain
2 fused to a HER-2/neu phosphorylation domain, wherein the protein is capable of
3 producing an immune response in a warm-blooded animal.
- 1 2. The protein of claim 1, wherein the protein has a sequence at least
2 80% identical to the sequence of SEQ ID NO:6, or wherein the protein comprises a
3 sequence at least 80% identical to the sequence of SEQ ID NO:3 fused to a sequence at
4 least 80% identical to the sequence of SEQ ID NO:4.
- 1 3. The protein of claim 1, wherein the protein comprises a sequence at
2 least 80 % identical to the sequence of SEQ ID NO:3 directly fused to an amino acid
3 sequence at least 80% identical to the sequence inclusive of Gln 991 to Val 1256 of SEQ
4 ID NO:2, or wherein the protein comprises a sequence at least 80 % identical to the
5 sequence of SEQ ID NO:3 fused to the amino acid sequence at least 80% identical to the
6 sequence inclusive of Gln 991 to Val 1256 of SEQ ID NO:2.
- 1 4. The protein of claim 1, wherein the protein comprises a sequence at
2 least 80% identical to the sequence of SEQ ID NO:8 directly fused to a sequence at least
3 80% identical to the sequence of SEQ ID NO:4, or wherein the protein comprises a
4 sequence at least 80% identical to the sequence of SEQ ID NO:8 fused to a sequence at
5 least 80% identical to the sequence of SEQ ID NO:4.
- 1 5. The protein of claim 1, wherein the protein comprises a sequence at
2 least 80% identical to the sequence of SEQ ID NO:8 directly fused to the amino acid
3 sequence inclusive of Gln 991 to Val 1256 of SEQ ID NO:2, or wherein the protein
4 comprises a sequence at least 80% identical to the sequence of SEQ ID NO:8 fused to a
5 sequence at least 80% identical to the amino acid sequence inclusive of Gln 991 to Val
6 1256 of SEQ ID NO:2.
- 1 6. The protein of claim 1, wherein the HER-2/neu extracellular
2 domain is fused to the HER-2/neu phosphorylation domain via a chemical linker.
- 1 7. The protein of claim 6, wherein the chemical linker is an amino
2 acid linker.

- 1 8. A nucleic acid molecule encoding the protein of claim 1.
- 1 9. A viral vector comprising a polynucleotide sequence encoding the
2 protein of claim 1.
- 1 10. A pharmaceutical composition comprising the protein of claim 1,
2 and a pharmaceutically acceptable carrier or diluent.
- 1 11. The pharmaceutical composition of claim 10, wherein the
2 pharmaceutical composition is a vaccine.
- 1 12. The pharmaceutical composition of claim 10, further comprising an
2 immunostimulatory substance.
- 1 13. The pharmaceutical composition of claim 12, wherein the protein is
2 presented in an oil-in-water emulsion.
- 1 14. The pharmaceutical composition of claim 12, wherein the
2 immunostimulatory substance is SBAS2, 3D-MPL, QS21, or a combination of 3D-MPL
3 and QS21.
- 1 15. A pharmaceutical composition comprising the nucleic acid
2 molecule of claim 8, and a pharmaceutically acceptable carrier or diluent.
- 1 16. The pharmaceutical composition of claim 15, wherein the
2 pharmaceutical composition is a vaccine.
- 1 17. The pharmaceutical composition of claim 15, further comprising an
2 immunostimulatory substance.
- 1 18. The pharmaceutical composition of claim 15, wherein the nucleic
2 acid molecule is a DNA molecule.
- 1 19. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the protein of claim 1 in an amount effective to elicit or enhance the immune response.

1 20. The method of claim 19, wherein the protein is administered in the
2 form of a vaccine.

1 21. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the nucleic acid molecule of claim 8 in an amount effective to elicit or enhance the
4 immune response.

1 22. The method of claim 21, wherein the nucleic acid molecule is in
2 the form of a vaccine.

1 23. The method of claim 21, wherein the step of administering
2 comprises transfecting cells of the warm-blooded animal *ex vivo* with the nucleic acid
3 molecule and subsequently delivering the transfected cells to the warm-blooded animal.

1 24. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the viral vector of claim 9 in an amount effective to elicit or enhance the immune
4 response.

1 25. The method of claim 24, wherein the step of administering
2 comprises infecting cells of the warm-blooded animal *ex vivo* with the viral vector and
3 subsequently delivering the infected cells to the warm-blooded animal.

1 26. An isolated protein comprising a HER-2/neu extracellular domain
2 fused to a fragment of the HER-2/neu phosphorylation domain, wherein the protein is
3 capable of producing an immune response in a warm-blooded animal.

1 27. The protein of claim 26, wherein the protein has a sequence at least
2 80% identical to the sequence of SEQ ID NO:7, or wherein the protein comprises a
3 sequence at least 80% identical to the sequence of SEQ ID NO:3 fused to a sequence at
4 least 80% identical to the sequence of SEQ ID NO:5.

1 28. The protein of claim 26, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:3 directly fused to a sequence at
3 least 80% identical to the amino acid sequence inclusive of Gln 991 to Arg 1049 of SEQ

4 ID NO:2, or wherein the protein comprises a sequence at least 80% identical to the
5 sequence of SEQ ID NO:3 fused to a sequence at least 80% identical to the amino acid
6 sequence inclusive of Gln 991 to Arg 1049 of SEQ ID NO:2.

1 29. The protein of claim 26, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:8 directly fused to a sequence at
3 least 80% identical to the sequence of SEQ ID NO:5, or wherein the protein comprises a
4 sequence at least 80% identical to the sequence of SEQ ID NO:8 fused to a sequence at
5 least 80% identical to the sequence of SEQ ID NO:5.

1 30. The protein of claim 26, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:8 directly fused to a sequence at
3 least 80% identical to the amino acid sequence inclusive of Gln 991 to Arg 1049 of SEQ
4 ID NO:2, or wherein the protein comprises a sequence at least 80% identical to the
5 sequence of SEQ ID NO:8 fused to a sequence at least 80% identical to the amino acid
6 sequence inclusive of Gln 991 to Arg 1049 of SEQ ID NO:2.

1 31. The protein of claim 26, wherein the HER-2/neu extracellular
2 domain is fused to the fragment of the HER-2/neu phosphorylation domain via a chemical
3 linker.

1 32. The protein of claim 31, wherein the chemical linker is an amino
2 acid linker.

1 33. A nucleic acid molecule encoding the protein of claim 26.

1 34. A viral vector comprising a polynucleotide sequence encoding the
2 protein of claim 26.

1 35. A pharmaceutical composition comprising the protein of claim 26,
2 and a pharmaceutically acceptable carrier or diluent.

1 36. The pharmaceutical composition of claim 35, wherein the
2 pharmaceutical composition is a vaccine.

1 37. The pharmaceutical composition of claim 35, further comprising an
2 immunostimulatory substance.

1 38. The pharmaceutical composition of claim 37, wherein the protein is
2 presented in an oil-in-water emulsion.

1 39. The pharmaceutical composition of claim 37, wherein the
2 immunostimulatory substance is SBAS2, 3D-MPL, QS21, or a combination of 3D-MPL
3 and QS21.

1 40. A pharmaceutical composition comprising the nucleic acid
2 molecule of claim 33, and a pharmaceutically acceptable carrier or diluent.

1 41. The pharmaceutical composition of claim 40, wherein the
2 pharmaceutical composition is a vaccine.

1 42. The pharmaceutical composition of claim 40, further comprising an
2 immunostimulatory substance.

1 43. The pharmaceutical composition of claim 40, wherein the nucleic
2 acid molecule is a DNA molecule.

1 44. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the protein of claim 26 in an amount effective to elicit or enhance the immune response.

1 45. The method of claim 44, wherein the protein is administered in the
2 form of a vaccine.

1 46. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the nucleic acid molecule of claim 33 in an amount effective to elicit or enhance the
4 immune response.

1 47. The method of claim 46, wherein the nucleic acid molecule is in
2 the form of a vaccine.

1 48. The method of claim 46, wherein the step of administering
2 comprises transfecting cells of the warm-blooded animal *ex vivo* with the nucleic acid
3 molecule and subsequently delivering the transfected cells to the warm-blooded animal.

1 49. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the viral vector of claim 34 in an amount effective to elicit or enhance the immune
4 response.

1 50. The method of claim 49, wherein the step of administering
2 comprises infecting cells of the warm-blooded animal *ex vivo* with the viral vector and
3 subsequently delivering the infected cells to the warm-blooded animal.

1 51. An isolated protein comprising a HER-2/neu extracellular domain
2 fused to a HER-2/neu intracellular domain, wherein the protein is capable of producing an
3 immune response in a warm-blooded animal.

1 52. The protein of claim 51, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:3 fused directly to a sequence at
3 least 80% identical to the amino acid sequence inclusive of Lys 676 to Val 1255 in SEQ
4 ID NO:1, or wherein the protein comprises a sequence at least 80% identical to the
5 sequence of SEQ ID NO:3 fused to a sequence at least 80% identical to the amino acid
6 sequence inclusive of Lys 676 to Val 1255 of SEQ ID NO:1 via at least one of a chemical
7 or amino acid linking group.

1 53. The protein of claim 51, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:3 directly fused to a sequence at
3 least 80% identical to the amino acid sequence inclusive of Lys 677 to Val 1256 of SEQ
4 ID NO:2, or wherein the protein comprises a sequence at least 80% identical to the
5 sequence of SEQ ID NO:3 fused to a sequence at least 80% identical to the amino acid
6 sequence inclusive of Lys 677 to Val 1256 of SEQ ID NO:2 via at least one of a chemical
7 or amino acid linking group.

1 54. The protein of claim 51, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:8 directly fused to a sequence at
3 least 80% identical to the amino acid sequence inclusive of Lys 676 to Val 1255 of SEQ
4 ID NO:1, or wherein the protein comprises a sequence at least 80% identical to the
5 sequence of SEQ ID NO:8 fused to a sequence at least 80% identical to the amino acid

6 sequence inclusive of Lys 676 to Val 1255 of SEQ ID NO:1 via at least one of a chemical
7 or amino acid linking group.

1 55. The protein of claim 51, wherein the protein comprises a sequence
2 at least 80% identical to the sequence of SEQ ID NO:8 directly fused to a sequence at
3 least 80% identical to the amino acid sequence inclusive of Lys 677 to Val 1256 of SEQ
4 ID NO:2, or wherein the protein comprises a sequence at least 80% identical to the
5 sequence of SEQ ID NO:8 fused to a sequence at least 80% identical to the amino acid
6 sequence inclusive of Lys 677 to Val 1256 of SEQ ID NO:2 via at least one of a chemical
7 or amino acid linking group.

1 56. The protein of claim 51, wherein the HER-2/neu extracellular
2 domain is fused to the HER-2/neu intracellular domain via a chemical linker.

1 57. The protein of claim 56, wherein the chemical linker is an amino
2 acid linker.

1 58. A nucleic acid molecule encoding the protein of claim 51.

1 59. A viral vector comprising a polynucleotide sequence encoding the
2 protein of claim 51.

1 60. A pharmaceutical composition comprising the protein of claim 51,
2 and a pharmaceutically acceptable carrier or diluent.

1 61. The pharmaceutical composition of claim 60, wherein the
2 pharmaceutical composition is a vaccine.

1 62. The pharmaceutical composition of claim 60, further comprising an
2 immunostimulatory substance.

1 63. The pharmaceutical composition of claim 62, wherein the protein is
2 presented in an oil-in-water emulsion.

1 64. The pharmaceutical composition of claim 62, wherein the
2 immunostimulatory substance is SBAS2, 3D-MPL, QS21, or a combination of 3D-MPL
3 and QS21.

1 65. A pharmaceutical composition comprising the nucleic acid
2 molecule of claim 58, and a pharmaceutically acceptable carrier or diluent.

1 66. The pharmaceutical composition of claim 65, wherein the
2 pharmaceutical composition is a vaccine.

1 67. The pharmaceutical composition of claim 65, further comprising an
2 immunostimulatory substance.

1 68. The pharmaceutical composition of claim 65, wherein the nucleic
2 acid molecule is a DNA molecule.

1 69. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the protein of claim 51 in an amount effective to elicit or enhance the immune response.

1 70. The method of claim 69, wherein the protein is administered in the
2 form of a vaccine.

1 71. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the nucleic acid molecule of claim 58 in an amount effective to elicit or enhance the
4 immune response.

1 72. The method of claim 71, wherein the nucleic acid molecule is in
2 the form of a vaccine.

1 73. The method of claim 71, wherein the step of administering
2 comprises transfecting cells of the warm-blooded animal *ex vivo* with the nucleic acid
3 molecule and subsequently delivering the transfected cells to the warm-blooded animal.

1 74. A method for eliciting or enhancing an immune response to HER-
2 2/neu protein, the method comprising the step of administering to a warm-blooded animal
3 the viral vector of claim 59 in an amount effective to elicit or enhance the immune
4 response.

1 75. The method of claim 74, wherein the step of administering
2 comprises infecting cells of the warm-blooded animal *ex vivo* with the viral vector and
3 subsequently delivering the infected cells to the warm-blooded animal.

1 76. A method for inhibiting the development of a cancer in a patient,
2 the method comprising the step of administering to a patient an effective amount of a
3 fusion polypeptide according to claim 1, 26, or 51 and thereby inhibiting the development
4 of a cancer in the patient.

1 77. A method for inhibiting the development of a cancer in a patient,
2 the method comprising the step of administering to a patient an effective amount of a
3 polynucleotide according to claim 8, 33, or 58 and thereby inhibiting the development of
4 a cancer in the patient.

1 78. A method for inhibiting the development of a cancer in a patient,
2 the method comprising the step of administering to a patient an effective amount of an
3 antigen-presenting cell that expresses a fusion polypeptide according to claim 1, 26, or
4 51, and thereby inhibiting the development of a cancer in the patient.

1 79. A method according to claim 78, wherein the antigen-presenting
2 cell is a dendritic cell.

1 80. A method according to any one of claims 76-79, wherein the
2 cancer is breast, ovarian, colon, lung or prostate cancer.

1 81. A method for removing tumor cells from a biological sample, the
2 method comprising the step of contacting a biological sample with T cells that
3 specifically react with a HER-2/neu fusion protein, wherein the fusion protein comprises
4 an amino acid sequence that is encoded by a polynucleotide sequence selected from the
5 group consisting of:

6 (i) polynucleotides recited in any one of SEQ ID NO:8, 33, or
7 58; and

8 (ii) complements of the foregoing polynucleotides;

9 wherein the step of contacting is performed under conditions and for a
10 time sufficient to permit the removal of cells expressing the antigen from the sample.

1 82. A method according to claim 81, wherein the biological sample is
2 blood or a fraction thereof.

1 83. A method for inhibiting the development of a cancer in a patient,
2 comprising the step of administering to a patient a biological sample treated according to
3 the method of claim 81.

1 84. A method for stimulating and/or expanding T cells specific for a
2 HER-2/neu fusion protein, the method comprising the step of contacting T cells with one
3 or more of:

- 4 (i) a fusion protein according to claims 1, 26, or 51;
5 (ii) a polynucleotide encoding such a fusion protein; or
6 (iii) an antigen presenting cell that expresses such a fusion protein;
7 under conditions and for a time sufficient to permit the stimulation and/or
8 expansion of T cells.

1 85. An isolated T cell population, comprising T cells prepared
2 according to the method of claim 84.

1 86. A method for inhibiting the development of a cancer in a patient,
2 the method comprising the step of administering to a patient an effective amount of a T
3 cell population according to claim 85.

1 87. A method for inhibiting the development of a cancer in a patient,
2 the method comprising the steps of:

3 (a) incubating CD4⁺ and/or CD8⁺ T cells isolated from a patient with
4 at least one component selected from the group consisting of:

- 5 (i) a fusion protein according to claims 1, 26, or 51;
6 (ii) a polynucleotide encoding such a fusion protein; and
7 (iii) an antigen-presenting cell that expresses such a fusion
8 protein;

9 such that T cells proliferate; and

10 (b) administering to the patient an effective amount of the proliferated
11 T cells, thereby inhibiting the development of a cancer in the patient.

1 88. A method for inhibiting the development of a cancer in a patient,
2 the method comprising the steps of:

3 (a) incubating CD4⁺ and/or CD8⁺ T cells isolated from a patient with
4 at least one component selected from the group consisting of:

5 (i) a fusion protein according to claims 1, 26, or 51;
6 (ii) a polynucleotide encoding such a fusion protein; and
7 (iii) an antigen-presenting cell that expresses such a fusion
8 protein;

9 such that T cells proliferate;

10 (b) cloning at least one proliferated cell; and

11 (c) administering to the patient an effective amount of the cloned T
12 cells, thereby inhibiting the development of a cancer in the patient.

1 89. A method of making a fusion protein according to claims 1, 26, or
2 51, the method comprising the steps of:

3 (a) introducing into a cell an expression vector comprising a
4 polynucleotide according to claims 8, 33, or 58;

5 (b) culturing the transfected cell; and

6 (c) purifying the expressed protein.

1 90. The method of claim 89, wherein the cell is a CHO cell.

1 91. The method of claim 89, wherein the cell is cultured in suspension,
2 under serum-free conditions.

1 92. The method of claim 89, wherein the expressed protein is purified
2 by a two-step procedure, the procedure comprising:

3 (a) anion exchange chromatography on Q sepharose High Performance
4 Columns; and

5 (b) hydrophobic chromatography on Phenyl Sepharose 6 Fast Flow
6 low substitution.

HER-2/NEU FUSION PROTEINS

ABSTRACT OF THE DISCLOSURE

The present invention is generally directed to HER-2/neu fusion proteins, nucleic acid molecules encoding HER-2/neu fusion proteins, viral vectors expressing HER-2/neu fusion proteins, and pharmaceutical compositions (*e.g.*, vaccines) comprising the HER-2/neu fusion proteins and/or nucleic acid molecules encoding the HER-2/neu fusion proteins. The present invention is also directed to methods of treating or preventing cancer by eliciting or enhancing an immune response to the HER-2/neu protein, including for uses in the treatment of malignancies associated with the HER-2/neu oncogene.

SF 1058004 v2

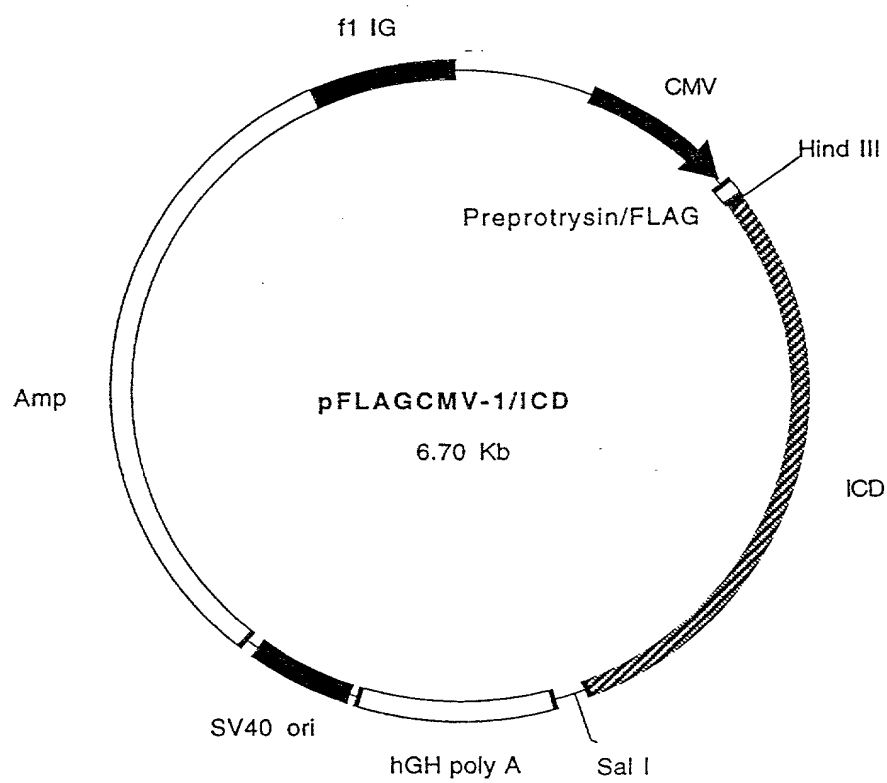


Fig. 1

Fig. 2

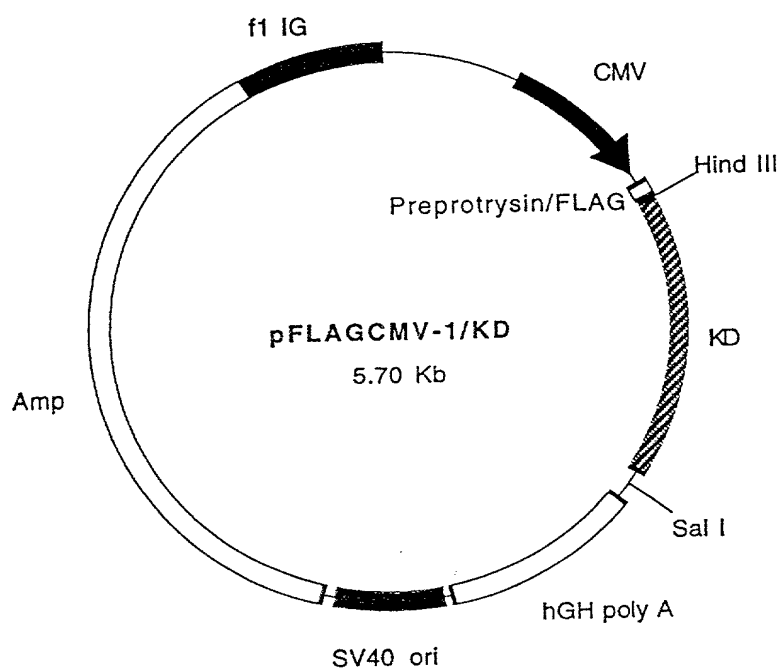


Fig. 3

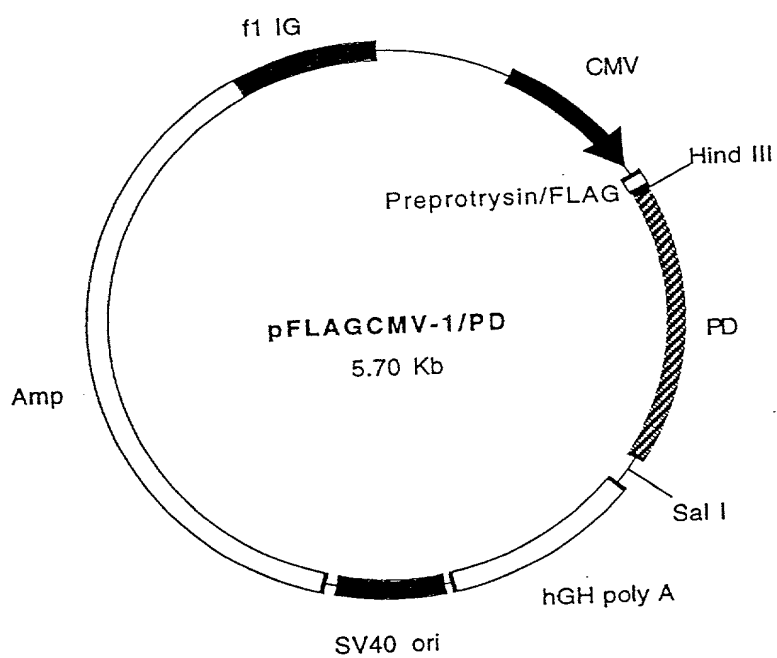
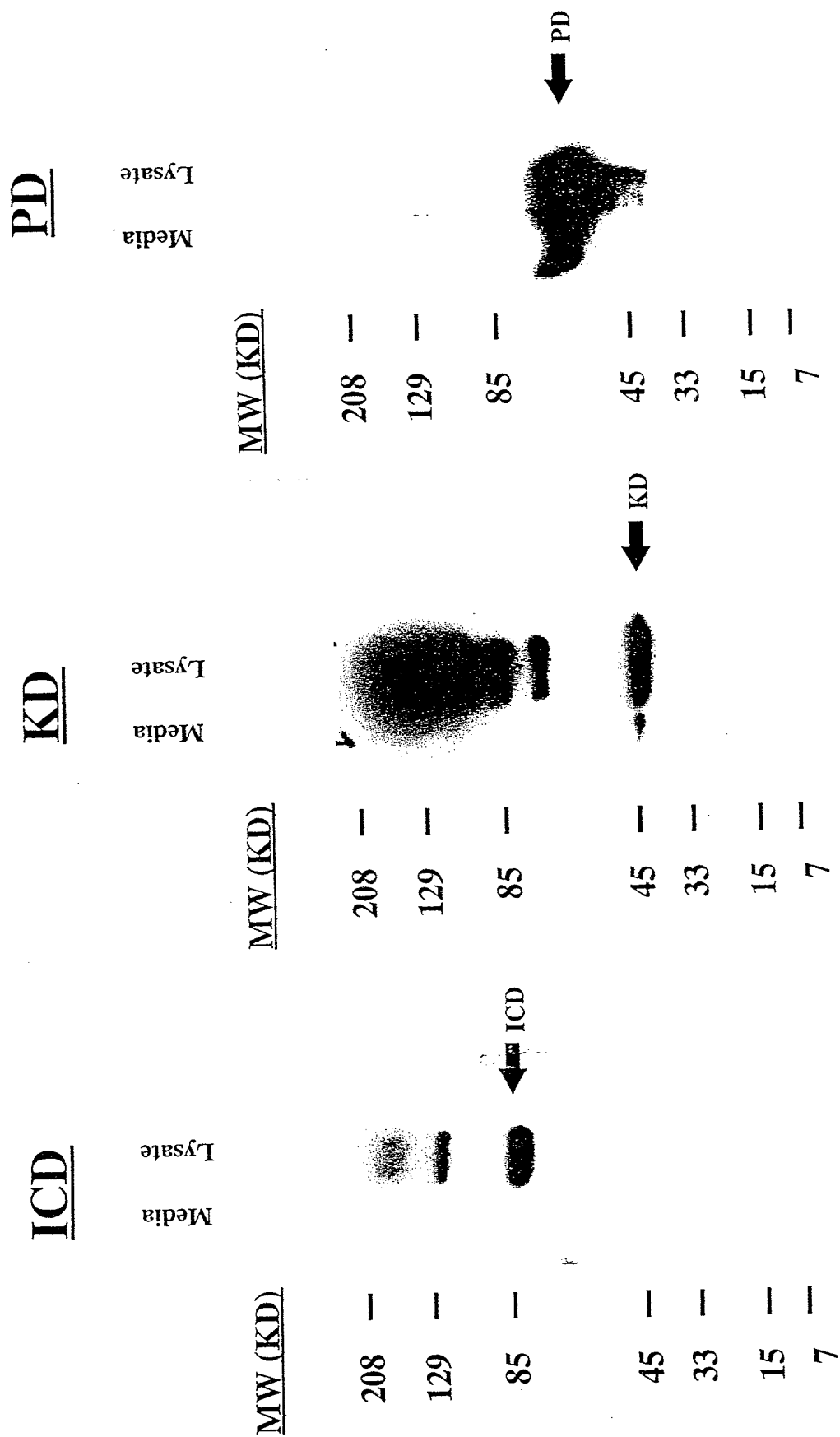


Fig. 4



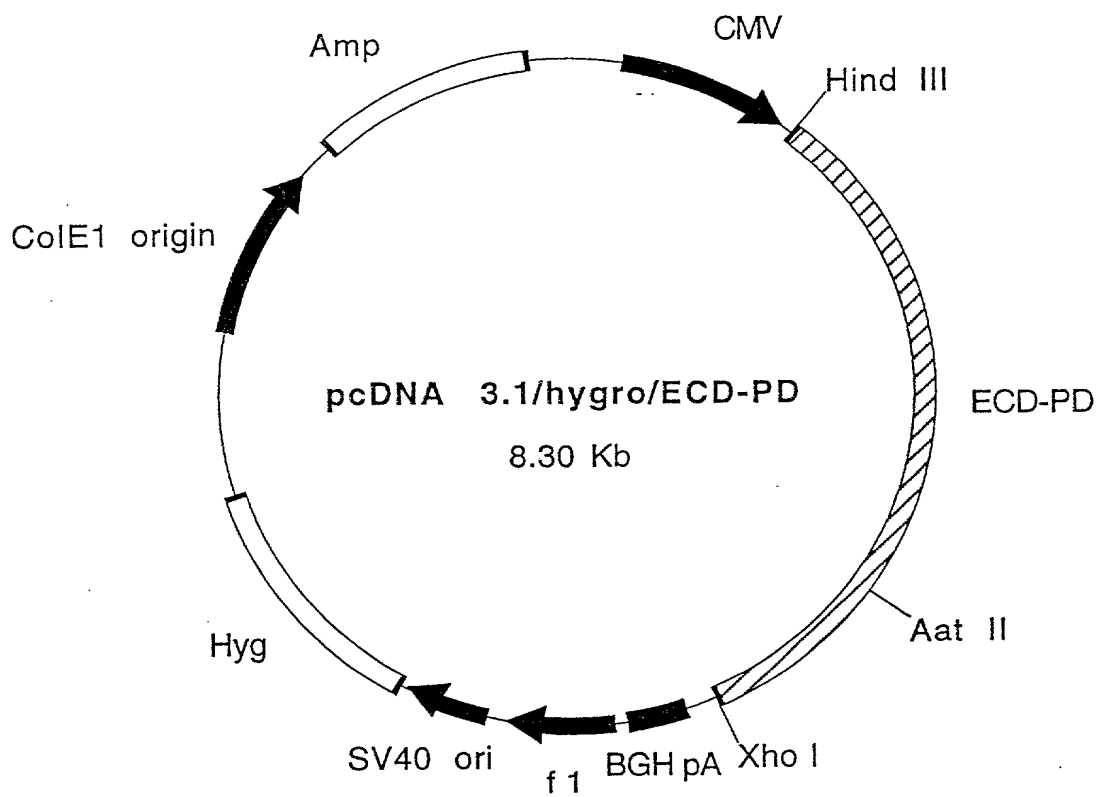


Fig. 5

pcDNA3.1hyg/ECD-PD expression

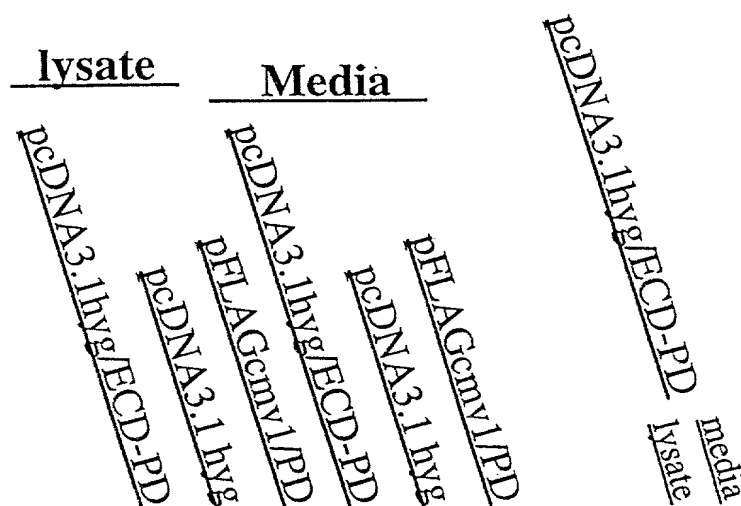
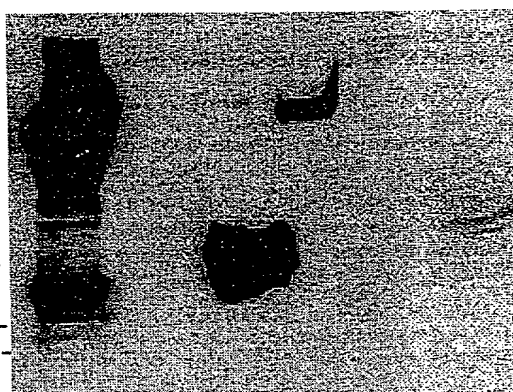


Fig. 6A

Her2/neu AB-3

293T

203-
116-
83-
49-
37-
28-



anti ECD



Fig. 6B

Her2/neu AB-3

CHO

203-
116-
83-
49-
37-
28-



anti ECD



Fig. 7 (SEQ ID NO: 1)

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|

Fig. 7 (SEQ ID NO: 1)

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
| 610 | | | | | | | | | | | | | | | | | | | 620 | | | | | | | | | | | | | | | | | | |
| Pro | Ser | Gly | Val | Lys | Pro | Asp | Leu | Ser | Tyr | Met | Pro | Ile | Trp | Lys | Phe | Pro | Asp | Glu | Glu | 620 | | | | | | | | | | | | | | | | | |
| Gly | Ala | Cys | Gln | Pro | Cys | Pro | Ile | Asn | Cys | Thr | His | Ser | Cys | Val | Asp | Leu | Asp | Asp | Lys | 640 | | | | | | | | | | | | | | | | | |
| Gly | Cys | Pro | Ala | Glu | Gln | Arg | Ala | Ser | Pro | Leu | Thr | Ser | Ile | Ile | Ser | Ala | Val | Val | Gly | 660 | | | | | | | | | | | | | | | | | |
| Ile | Leu | Leu | Val | Val | Val | Leu | Gly | Val | Val | Phe | Gly | Ile | Leu | Ile | Lys | Arg | Arg | Gln | Gln | 680 | | | | | | | | | | | | | | | | | |
| Lys | Ile | Arg | Lys | Tyr | Thr | Met | Arg | Arg | Leu | Leu | Gln | Glu | Thr | Glu | Leu | Val | Glu | Pro | Leu | 700 | | | | | | | | | | | | | | | | | |
| 710 | | | | | | | | | | | | | | | | | | | 720 | | | | | | | | | | | | | | | | | | |
| Thr | Pro | Ser | Gly | Ala | Met | Pro | Asn | Gln | Ala | Gln | Met | Arg | Ile | Leu | Lys | Glu | Thr | Glu | Leu | 720 | | | | | | | | | | | | | | | | | |
| Arg | Lys | Val | Lys | Val | Leu | Gly | Ser | Gly | Ala | Phe | Gly | Thr | Val | Tyr | Lys | Gly | Ile | Trp | Ile | 740 | | | | | | | | | | | | | | | | | |
| Pro | Asp | Gly | Glu | Asn | Val | Lys | Ile | Pro | Val | Ala | Ile | Lys | Val | Leu | Arg | Glu | Asn | Thr | Ser | 760 | | | | | | | | | | | | | | | | | |
| Pro | Lys | Ala | Asn | Lys | Glu | Ile | Leu | Asp | Glu | Ala | Tyr | Val | Met | Ala | Gly | Val | Gly | Ser | Pro | 780 | | | | | | | | | | | | | | | | | |
| Tyr | Val | Ser | Arg | Leu | Leu | Gly | Ile | Cys | Leu | Thr | Ser | Thr | Val | Gln | Leu | Val | Thr | Gln | Leu | 800 | | | | | | | | | | | | | | | | | |
| 810 | | | | | | | | | | | | | | | | | | | 820 | | | | | | | | | | | | | | | | | | |
| Met | Pro | Tyr | Gly | Cys | Leu | Leu | Asp | His | Val | Arg | Glu | Asn | Arg | Gly | Arg | Leu | Gly | Ser | Gln | 820 | | | | | | | | | | | | | | | | | |
| Asp | Leu | Leu | Asn | Trp | Cys | Met | Gln | Ile | Ala | Lys | Gly | Met | Ser | Tyr | Leu | Glu | Asp | Val | Arg | 840 | | | | | | | | | | | | | | | | | |
| Leu | Val | His | Arg | Asp | Leu | Ala | Ala | Arg | Asn | Val | Leu | Val | Lys | Ser | Pro | Asn | His | Val | Lys | 860 | | | | | | | | | | | | | | | | | |
| Ile | Thr | Asp | Phe | Gly | Leu | Ala | Arg | Leu | Leu | Asp | Ile | Asp | Glu | Thr | Glu | Tyr | His | Ala | Asp | 880 | | | | | | | | | | | | | | | | | |
| Gly | Gly | Lys | Val | Pro | Ile | Lys | Trp | Met | Ala | Leu | Glu | Ser | Ile | Leu | Arg | Arg | Arg | Phe | Thr | 900 | | | | | | | | | | | | | | | | | |
| 910 | | | | | | | | | | | | | | | | | | | 920 | | | | | | | | | | | | | | | | | | |
| His | Gln | Ser | Asp | Val | Trp | Ser | Tyr | Gly | Val | Thr | Val | Trp | Glu | Leu | Met | Thr | Phe | Gly | Ala | 920 | | | | | | | | | | | | | | | | | |
| Lys | Pro | Tyr | Asp | Gly | Ile | Pro | Ala | Arg | Glu | Ile | Pro | Asp | Leu | Leu | Glu | Lys | Gly | Glu | Arg | 940 | | | | | | | | | | | | | | | | | |
| Leu | Pro | Gln | Pro | Pro | Ile | Cys | Thr | Ile | Asp | Val | Tyr | Met | Ile | Met | Val | Lys | Cys | Trp | Met | 960 | | | | | | | | | | | | | | | | | |
| Ile | Asp | Ser | Glu | Cys | Arg | Pro | Arg | Phe | Arg | Glu | Leu | Val | Ser | Glu | Phe | Ser | Arg | Met | Ala | 980 | | | | | | | | | | | | | | | | | |
| Arg | Asp | Pro | Gln | Arg | Phe | Val | Val | Ile | Gln | Asn | Glu | Asp | Leu | Gly | Pro | Ala | Ser | Pro | Leu | 1000 | | | | | | | | | | | | | | | | | |
| 1010 | | | | | | | | | | | | | | | | | | | 1020 | | | | | | | | | | | | | | | | | | |
| Asp | Ser | Thr | Phe | Tyr | Arg | Ser | Leu | Leu | Glu | Asp | Asp | Asp | Met | Gly | Asp | Leu | Val | Asp | Ala | 1020 | | | | | | | | | | | | | | | | | |
| Glu | Glu | Tyr | Leu | Val | Pro | Gln | Gln | Gly | Phe | Phe | Cys | Pro | Asp | Pro | Ala | Pro | Gly | Ala | Gly | 1040 | | | | | | | | | | | | | | | | | |
| Gly | Met | Val | His | His | Arg | His | Arg | Ser | Ser | Ser | Thr | Arg | Ser | Gly | Gly | Gly | Asp | Leu | Thr | 1060 | | | | | | | | | | | | | | | | | |
| Leu | Gly | Leu | Glu | Pro | Ser | Glu | Glu | Glu | Ala | Pro | Arg | Ser | Pro | Leu | Ala | Pro | Ser | Glu | Gly | 1080 | | | | | | | | | | | | | | | | | |
| Ala | Gly | Ser | Asp | Val | Phe | Asp | Gly | Asp | Leu | Gly | Met | Gly | Ala | Ala | Lys | Gly | Leu | Gln | Ser | 1100 | | | | | | | | | | | | | | | | | |
| 1110 | | | | | | | | | | | | | | | | | | | 1120 | | | | | | | | | | | | | | | | | | |
| Leu | Pro | Thr | His | Asp | Pro | Ser | Pro | Leu | Gln | Arg | Tyr | Ser | Glu | Asp | Pro | Thr | Val | Pro | Leu | 1120 | | | | | | | | | | | | | | | | | |
| Pro | Ser | Glu | Thr | Asp | Gly | Tyr | Val | Ala | Pro | Leu | Thr | Cys | Ser | Pro | Gln | Pro | Glu | Tyr | Val | 1140 | | | | | | | | | | | | | | | | | |
| Asn | Gln | Pro | Asp | Val | Arg | Pro | Gln | Pro | Pro | Ser | Pro | Arg | Glu | Gly | Pro | Leu | Pro | Ala | Ala | 1160 | | | | | | | | | | | | | | | | | |
| Arg | Pro | Ala | Gly | Ala | Thr | Leu | Glu | Arg | Pro | Lys | Thr | Leu | Ser | Pro | Gly | Lys | Asn | Gly | Val | 1180 | | | | | | | | | | | | | | | | | |
| Val | Lys | Asp | Val | Phe | Ala | Phe | Gly | Gly | Ala | Val | Glu | Asn | Pro | Glu | Tyr | Leu | Thr | Pro | Gln | 1200 | | | | | | | | | | | | | | | | | |
| 1210 | | | | | | | | | | | | | | | | | | | 1220 | | | | | | | | | | | | | | | | | | |
| Gly | Gly | Ala | Ala | Pro | Gln | Pro | His | Pro | Pro | Pro | Ala | Phe | Ser | Pro | Ala | Phe | Asp | Asn | Leu | 1220 | | | | | | | | | | | | | | | | | |
| Tyr | Tyr | Trp | Asp | Gln | Asp | Pro | Pro | Glu | Arg | Gly | Ala | Pro | Pro | Ser | Thr | Phe | Lys | Gly | Thr | 1240 | | | | | | | | | | | | | | | | | |
| Pro | Thr | Ala | Glu | Asn | Pro | Glu | Tyr | Leu | Gly | Leu | Asp | Val | Pro | Val | . | . | . | . | 1257 | | | | | | | | | | | | | | | | | | |

Fig. 8 (SEQ ID NO: 2)

| | | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| Met | Glu | Leu | Ala | Ala | Trp | Cys | Arg | Trp | Gly | Phe | Leu | Leu | Ala | Leu | Leu | Pro | Pro | Gly | Ile | 20 | |
| Ala | Gly | Thr | Gln | Val | Cys | Thr | Gly | Thr | Asp | Met | Lys | Leu | Arg | Leu | Pro | Ala | Ser | Pro | Glu | 40 | |
| Thr | His | Leu | Asp | Met | Leu | Arg | His | Leu | Tyr | Gln | Gly | Cys | Gln | Val | Val | Gln | Gly | Asn | Leu | 60 | |
| Glu | Leu | Thr | Tyr | Val | Pro | Ala | Asn | Ala | Ser | Leu | Ser | Phe | Leu | Gln | Asp | Ile | Gln | Glu | Val | 80 | |
| Gln | Gly | Tyr | Met | Leu | Ile | Ala | His | Asn | Gln | Val | Lys | Arg | Val | Pro | Leu | Gln | Arg | Leu | Arg | 100 | |
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| Ile | Val | Arg | Gly | Thr | Gln | Leu | Phe | Glu | Asp | Lys | Tyr | Ala | Leu | Ala | Val | Leu | Asp | Asn | Arg | 120 | |
| Asp | Pro | Gln | Asp | Asn | Val | Ala | Ala | Ser | Thr | Pro | Gly | Arg | Thr | Pro | Glu | Gly | Leu | Arg | Glu | 140 | |
| Leu | Gln | Leu | Arg | Ser | Leu | Thr | Glu | Ile | Leu | Lys | Gly | Gly | Val | Leu | Ile | Arg | Gly | Asn | Pro | 160 | |
| Gln | Leu | Cys | Tyr | Gln | Asp | Met | Val | Leu | Trp | Lys | Asp | Val | Phe | Arg | Lys | Asn | Asn | Gln | Leu | 180 | |
| Ala | Pro | Val | Asp | Ile | Asp | Thr | Asn | Arg | Ser | Arg | Ala | Cys | Pro | Pro | Cys | Ala | Pro | Ala | Cys | 200 | |
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| Lys | Asp | Asn | His | Cys | Trp | Gly | Glu | Ser | Pro | Glu | Asp | Cys | Gln | Ile | Leu | Thr | Gly | Thr | Ile | 220 | |
| Cys | Thr | Ser | Gly | Cys | Ala | Arg | Cys | Lys | Gly | Arg | Leu | Pro | Thr | Asp | Cys | Cys | His | Glu | Gln | 240 | |
| Cys | Ala | Ala | Gly | Cys | Thr | Gly | Pro | Lys | His | Ser | Asp | Cys | Leu | Ala | Cys | Leu | His | Phe | Asn | 260 | |
| His | Ser | Gly | Ile | Cys | Glu | Leu | His | Cys | Pro | Ala | Leu | Val | Thr | Tyr | Asn | Thr | Asp | Thr | Phe | 280 | |
| Glu | Ser | Met | His | Asn | Pro | Glu | Gly | Arg | Tyr | Thr | Phe | Gly | Ala | Ser | Cys | Val | Thr | Thr | Cys | 300 | |
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| Pro | Tyr | Asn | Tyr | Leu | Ser | Thr | Glu | Val | Gly | Ser | Cys | Thr | Leu | Val | Cys | Pro | Pro | Asn | Asn | 320 | |
| Gln | Glu | Val | Thr | Ala | Glu | Asp | Gly | Thr | Gln | Arg | Cys | Glu | Lys | Cys | Ser | Lys | Pro | Cys | Ala | 340 | |
| Arg | Val | Cys | Tyr | Gly | Leu | Gly | Met | Glu | His | Leu | Arg | Gly | Ala | Arg | Ala | Ile | Thr | Ser | Asp | 360 | |
| Asn | Val | Gln | Glu | Phe | Asp | Gly | Cys | Lys | Lys | Ile | Phe | Gly | Ser | Leu | Ala | Phe | Leu | Pro | Glu | 380 | |
| Ser | Phe | Asp | Gly | Asp | Pro | Ser | Ser | Gly | Ile | Ala | Pro | Leu | Arg | Pro | Glu | Gln | Leu | Gln | Val | 400 | |
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| Phe | Glu | Thr | Leu | Glu | Glu | Ile | Thr | Gly | Tyr | Leu | Tyr | Ile | Ser | Ala | Trp | Pro | Asp | Ser | Leu | 420 | |
| Arg | Asp | Leu | Ser | Val | Phe | Gln | Asn | Leu | Arg | Ile | Ile | Arg | Gly | Arg | Ile | Leu | His | Asp | Gly | 440 | |
| Ala | Tyr | Ser | Leu | Thr | Leu | Gln | Gly | Leu | Gly | Ile | His | Ser | Leu | Gly | Leu | Arg | Ser | Leu | Arg | 460 | |
| Glu | Leu | Gly | Ser | Gly | Leu | Ala | Leu | Ile | His | Arg | Asn | Ala | His | Leu | Cys | Phe | Val | His | Thr | 480 | |
| Val | Pro | Trp | Asp | Gln | Leu | Phe | Arg | Asn | Pro | His | Gln | Ala | Leu | Leu | His | Ser | Gly | Asn | Arg | 500 | |
| | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | |
| Pro | Glu | Glu | Asp | Cys | Gly | Leu | Glu | Gly | Leu | Val | Cys | Asn | Ser | Leu | Cys | Ala | His | Gly | His | 520 | |
| Cys | Trp | Gly | Pro | Gly | Pro | Thr | Gln | Cys | Val | Asn | Cys | Ser | His | Phe | Leu | Arg | Gly | Gln | Glu | 540 | |
| Cys | Val | Glu | Glu | Cys | Arg | Val | Trp | Lys | Gly | Leu | Pro | Arg | Glu | Tyr | Val | Ser | Asp | Lys | Arg | 560 | |
| Cys | Leu | Pro | Cys | His | Pro | Glu | Cys | Gln | Pro | Gln | Asn | Ser | Ser | Glu | Thr | Cys | Phe | Gly | Ser | 580 | |
| Glu | Ala | Asp | Gln | Cys | Ala | Ala | Cys | Ala | His | Tyr | Lys | Asp | Ser | Ser | Ser | Cys | Val | Ala | Arg | 600 | |

Fig. 8 (SEQ ID NO: 2)

| | | | | | | | | | | | | | | | | | | | | |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|
| 610 | | | | | | | | | | | | | | | | | | | 620 | |
| Cys | Pro | Ser | Gly | Val | Lys | Pro | Asp | Leu | Ser | Tyr | Met | Pro | Ile | Trp | Lys | Tyr | Pro | Asp | Glu | 620 |
| Glu | Gly | Ile | Cys | Gln | Pro | Cys | Pro | Ile | Asn | Cys | Thr | His | Ser | Cys | Val | Asp | Leu | Asp | Glu | 640 |
| Arg | Gly | Cys | Pro | Ala | Glu | Gln | Arg | Ala | Ser | Pro | Val | Thr | Phe | Ile | Ile | Ala | Thr | Val | Val | 660 |
| Gly | Val | Leu | Leu | Phe | Leu | Ile | Leu | Val | Val | Val | Val | Gly | Ile | Leu | Ile | Lys | Arg | Arg | Arg | 680 |
| Gln | Lys | Ile | Arg | Lys | Tyr | Thr | Met | Arg | Arg | Leu | Leu | Gln | Glu | Thr | Glu | Leu | Val | Glu | Pro | 700 |
| 710 | | | | | | | | | | | | | | | | | | | 720 | |
| Leu | Thr | Pro | Ser | Gly | Ala | Met | Pro | Asn | Gln | Ala | Gln | Met | Arg | Ile | Leu | Lys | Glu | Thr | Glu | 720 |
| Leu | Arg | Lys | Val | Lys | Val | Leu | Gly | Ser | Gly | Ala | Phe | Gly | Thr | Val | Tyr | Lys | Gly | Ile | Trp | 740 |
| Ile | Pro | Asp | Gly | Glu | Asn | Val | Lys | Ile | Pro | Val | Ala | Ile | Lys | Val | Leu | Arg | Glu | Asn | Thr | 760 |
| Ser | Pro | Lys | Ala | Asn | Lys | Glu | Ile | Leu | Asp | Glu | Ala | Tyr | Val | Met | Ala | Gly | Val | Gly | Ser | 780 |
| Pro | Tyr | Val | Ser | Arg | Leu | Leu | Gly | Ile | Cys | Leu | Thr | Ser | Thr | Val | Gln | Leu | Val | Thr | Gln | 800 |
| 810 | | | | | | | | | | | | | | | | | | | 820 | |
| Leu | Met | Pro | Tyr | Gly | Cys | Leu | Leu | Asp | His | Val | Arg | Glu | His | Arg | Gly | Arg | Leu | Gly | Ser | 820 |
| Gln | Asp | Leu | Leu | Asn | Trp | Cys | Val | Gln | Ile | Ala | Lys | Gly | Met | Ser | Tyr | Leu | Glu | Asp | Val | 840 |
| Arg | Leu | Val | His | Arg | Asp | Leu | Ala | Ala | Arg | Asn | Val | Leu | Val | Lys | Ser | Pro | Asn | His | Val | 860 |
| Lys | Ile | Thr | Asp | Phe | Gly | Leu | Ala | Arg | Leu | Leu | Asp | Ile | Asp | Glu | Thr | Glu | Tyr | His | Ala | 880 |
| Asp | Gly | Gly | Lys | Val | Pro | Ile | Lys | Trp | Met | Ala | Leu | Glu | Ser | Ile | Leu | Arg | Arg | Arg | Phe | 900 |
| 910 | | | | | | | | | | | | | | | | | | | 920 | |
| Thr | His | Gln | Ser | Asp | Val | Trp | Ser | Tyr | Gly | Val | Thr | Val | Trp | Glu | Leu | Met | Thr | Phe | Gly | 920 |
| Ala | Lys | Pro | Tyr | Asp | Gly | Ile | Pro | Ala | Arg | Glu | Ile | Pro | Asp | Leu | Leu | Glu | Lys | Gly | Glu | 940 |
| Arg | Leu | Pro | Gln | Pro | Pro | Ile | Cys | Thr | Ile | Asp | Val | Tyr | Met | Ile | Met | Val | Lys | Cys | Trp | 960 |
| Met | Ile | Asp | Ser | Glu | Cys | Arg | Pro | Arg | Phe | Arg | Glu | Leu | Val | Ser | Glu | Phe | Ser | Arg | Met | 980 |
| Ala | Arg | Asp | Pro | Gln | Arg | Phe | Val | Val | Ile | Gln | Asn | Glu | Asp | Leu | Gly | Pro | Ser | Ser | Pro | 1000 |
| 1010 | | | | | | | | | | | | | | | | | | | 1020 | |
| Met | Asp | Ser | Thr | Phe | Tyr | Arg | Ser | Leu | Leu | Glu | Asp | Asp | Asp | Met | Gly | Asp | Leu | Val | Asp | 1020 |
| Ala | Glu | Glu | Tyr | Leu | Val | Pro | Gln | Gln | Gly | Phe | Phe | Ser | Pro | Asp | Pro | Thr | Pro | Gly | Thr | 1040 |
| Gly | Ser | Thr | Ala | His | Arg | Arg | His | Arg | Ser | Ser | Ser | Thr | Arg | Ser | Gly | Gly | Gly | Glu | Leu | 1060 |
| Thr | Leu | Gly | Leu | Glu | Pro | Ser | Glu | Glu | Gly | Pro | Pro | Arg | Ser | Pro | Leu | Ala | Pro | Ser | Glu | 1080 |
| Gly | Ala | Gly | Ser | Asp | Val | Phe | Asp | Gly | Asp | Leu | Ala | Met | Gly | Val | Thr | Lys | Gly | Leu | Gln | 1100 |
| 1110 | | | | | | | | | | | | | | | | | | | 1120 | |
| Ser | Leu | Ser | Pro | His | Asp | Leu | Ser | Pro | Leu | Gln | Arg | Tyr | Ser | Glu | Asp | Pro | Thr | Leu | Pro | 1120 |
| Leu | Pro | Pro | Glu | Thr | Asp | Gly | Tyr | Val | Ala | Pro | Leu | Ala | Cys | Ser | Pro | Gln | Pro | Glu | Tyr | 1140 |
| Val | Asn | Gln | Ser | Glu | Val | Gln | Pro | Gln | Pro | Pro | Leu | Thr | Pro | Glu | Gly | Pro | Leu | Pro | Pro | 1160 |
| Val | Arg | Pro | Ala | Gly | Ala | Thr | Leu | Glu | Arg | Pro | Lys | Thr | Leu | Ser | Pro | Gly | Lys | Asn | Gly | 1180 |
| Val | Val | Lys | Asp | Val | Phe | Ala | Phe | Gly | Gly | Ala | Val | Glu | Asn | Pro | Glu | Tyr | Leu | Val | Pro | 1200 |
| 1210 | | | | | | | | | | | | | | | | | | | 1220 | |
| Arg | Glu | Gly | Thr | Ala | Ser | Pro | Pro | His | Pro | Ser | Pro | Ala | Phe | Ser | Pro | Ala | Phe | Asp | Asn | 1220 |
| Leu | Tyr | Tyr | Trp | Asp | Gln | Asn | Ser | Ser | Glu | Gln | Gly | Pro | Pro | Pro | Ser | Asn | Phe | Glu | Gly | 1240 |
| Thr | Pro | Thr | Ala | Glu | Asn | Pro | Glu | Tyr | Leu | Gly | Leu | Asp | Val | Pro | Val | . | . | . | . | 1258 |

Fig. 9 (SEQ ID NO: 3)

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|--|--|--|--|--|--|--|-----|--|--|--|--|--|--|--|--|--|
| | | | | | | | | | | 10 | | | | | | | | | | | | | | | | | | | | 20 | | | | | | | | | |
| Met | Glu | Leu | Ala | Ala | Leu | Cys | Arg | Trp | Gly | Leu | Leu | Leu | Ala | Leu | Leu | Pro | Pro | Gly | Ala | 20 | | | | | | | | | | | | | | | | | | | |
| Ala | Ser | Thr | Gln | Val | Cys | Thr | Gly | Thr | Asp | Met | Lys | Leu | Arg | Leu | Pro | Ala | Ser | Pro | Glu | 40 | | | | | | | | | | | | | | | | | | | |
| Thr | His | Leu | Asp | Met | Leu | Arg | His | Leu | Tyr | Gln | Gly | Cys | Gln | Val | Val | Gln | Gly | Asn | Leu | 60 | | | | | | | | | | | | | | | | | | | |
| Glu | Leu | Thr | Tyr | Leu | Pro | Thr | Asn | Ala | Ser | Leu | Ser | Phe | Leu | Gln | Asp | Ile | Gln | Glu | Val | 80 | | | | | | | | | | | | | | | | | | | |
| Gln | Gly | Tyr | Val | Leu | Ile | Ala | His | Asn | Gln | Val | Arg | Gln | Val | Pro | Leu | Gln | Arg | Leu | Arg | 100 | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 110 | | | | | | | | | | | | | | | | | | | | 120 | | | | | | | | | |
| Ile | Val | Arg | Gly | Thr | Gln | Leu | Phe | Glu | Asp | Asn | Tyr | Ala | Leu | Ala | Val | Leu | Asp | Asn | Gly | 120 | | | | | | | | | | | | | | | | | | | |
| Asp | Pro | Leu | Asn | Asn | Thr | Thr | Pro | Val | Thr | Gly | Ala | Ser | Pro | Gly | Gly | Leu | Arg | Glu | Leu | 140 | | | | | | | | | | | | | | | | | | | |
| Gln | Leu | Arg | Ser | Leu | Thr | Glu | Ile | Leu | Lys | Gly | Gly | Val | Leu | Ile | Gln | Arg | Asn | Pro | Gln | 160 | | | | | | | | | | | | | | | | | | | |
| Leu | Cys | Tyr | Gln | Asp | Thr | Ile | Leu | Trp | Lys | Asp | Ile | Phe | His | Lys | Asn | Asn | Gln | Leu | Ala | 180 | | | | | | | | | | | | | | | | | | | |
| Leu | Thr | Leu | Ile | Asp | Thr | Asn | Arg | Ser | Arg | Ala | Cys | His | Pro | Cys | Ser | Pro | Met | Cys | Lys | 200 | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 210 | | | | | | | | | | | | | | | | | | | | 220 | | | | | | | | | |
| Gly | Ser | Arg | Cys | Trp | Gly | Glu | Ser | Ser | Glu | Asp | Cys | Gln | Ser | Leu | Thr | Arg | Thr | Val | Cys | 220 | | | | | | | | | | | | | | | | | | | |
| Ala | Gly | Gly | Cys | Ala | Arg | Cys | Lys | Gly | Pro | Leu | Pro | Thr | Asp | Cys | Cys | His | Glu | Gln | Cys | 240 | | | | | | | | | | | | | | | | | | | |
| Ala | Ala | Gly | Cys | Thr | Gly | Pro | Lys | His | Ser | Asp | Cys | Leu | Ala | Cys | Leu | His | Phe | Asn | His | 260 | | | | | | | | | | | | | | | | | | | |
| Ser | Gly | Ile | Cys | Glu | Leu | His | Cys | Pro | Ala | Leu | Val | Thr | Tyr | Asn | Thr | Asp | Thr | Phe | Glu | 280 | | | | | | | | | | | | | | | | | | | |
| Ser | Met | Pro | Asn | Pro | Glu | Gly | Arg | Tyr | Thr | Phe | Gly | Ala | Ser | Cys | Val | Thr | Ala | Cys | Pro | 300 | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 310 | | | | | | | | | | | | | | | | | | | | 320 | | | | | | | | | |
| Tyr | Asn | Tyr | Leu | Ser | Thr | Asp | Val | Gly | Ser | Cys | Thr | Leu | Val | Cys | Pro | Leu | His | Asn | Gln | 320 | | | | | | | | | | | | | | | | | | | |
| Glu | Val | Thr | Ala | Glu | Asp | Gly | Thr | Gln | Arg | Cys | Glu | Lys | Cys | Ser | Lys | Pro | Cys | Ala | Arg | 340 | | | | | | | | | | | | | | | | | | | |
| Val | Cys | Tyr | Gly | Leu | Gly | Met | Glu | His | Leu | Arg | Glu | Val | Arg | Ala | Val | Thr | Ser | Ala | Asn | 360 | | | | | | | | | | | | | | | | | | | |
| Ile | Gln | Glu | Phe | Ala | Gly | Cys | Lys | Lys | Ile | Phe | Gly | Ser | Leu | Ala | Phe | Leu | Pro | Glu | Ser | 380 | | | | | | | | | | | | | | | | | | | |
| Phe | Asp | Gly | Asp | Pro | Ala | Ser | Asn | Thr | Ala | Pro | Leu | Gln | Pro | Glu | Gln | Leu | Gln | Val | Phe | 400 | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 410 | | | | | | | | | | | | | | | | | | | | 420 | | | | | | | | | |
| Glu | Thr | Leu | Glu | Glu | Ile | Thr | Gly | Tyr | Leu | Tyr | Ile | Ser | Ala | Trp | Pro | Asp | Ser | Leu | Pro | 420 | | | | | | | | | | | | | | | | | | | |
| Asp | Leu | Ser | Val | Phe | Gln | Asn | Leu | Gln | Val | Ile | Arg | Gly | Arg | Ile | Leu | His | Asn | Gly | Ala | 440 | | | | | | | | | | | | | | | | | | | |
| Tyr | Ser | Leu | Thr | Leu | Gln | Gly | Leu | Gly | Ile | Ser | Trp | Leu | Gly | Leu | Arg | Ser | Leu | Arg | Glu | 460 | | | | | | | | | | | | | | | | | | | |
| Leu | Gly | Ser | Gly | Leu | Ala | Leu | Ile | His | His | Asn | Thr | His | Leu | Cys | Phe | Val | His | Thr | Val | 480 | | | | | | | | | | | | | | | | | | | |
| Pro | Trp | Asp | Gln | Leu | Phe | Arg | Asn | Pro | His | Gln | Ala | Leu | Leu | His | Thr | Ala | Asn | Arg | Pro | 500 | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 510 | | | | | | | | | | | | | | | | | | | | 520 | | | | | | | | | |
| Glu | Asp | Glu | Cys | Val | Gly | Glu | Gly | Leu | Ala | Cys | His | Gln | Leu | Cys | Ala | Arg | Gly | His | Cys | 520 | | | | | | | | | | | | | | | | | | | |
| Trp | Gly | Pro | Gly | Pro | Thr | Gln | Cys | Val | Asn | Cys | Ser | Gln | Phe | Leu | Arg | Gly | Gln | Glu | Cys | 540 | | | | | | | | | | | | | | | | | | | |
| Val | Glu | Glu | Cys | Arg | Val | Leu | Gln | Gly | Leu | Pro | Arg | Glu | Tyr | Val | Asn | Ala | Arg | His | Cys | 560 | | | | | | | | | | | | | | | | | | | |
| Leu | Pro | Cys | His | Pro | Glu | Cys | Gln | Pro | Gln | Asn | Gly | Ser | Val | Thr | Cys | Phe | Gly | Pro | Glu | 580 | | | | | | | | | | | | | | | | | | | |
| Ala | Asp | Gln | Cys | Val | Ala | Cys | Ala | His | Tyr | Lys | Asp | Pro | Pro | Phe | Cys | Val | Ala | Arg | Cys | 600 | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 610 | | | | | | | | | | | | | | | | | | | | 620 | | | | | | | | | |
| Pro | Ser | Gly | Val | Lys | Pro | Asp | Leu | Ser | Tyr | Met | Pro | Ile | Trp | Lys | Phe | Pro | Asp | Glu | Glu | 620 | | | | | | | | | | | | | | | | | | | |
| Gly | Ala | Cys | Gln | Pro | Cys | Pro | Ile | Asn | Cys | Thr | His | Ser | Cys | Val | Asp | Leu | Asp | Asp | Lys | 640 | | | | | | | | | | | | | | | | | | | |
| Gly | Cys | Pro | Ala | Glu | Gln | Arg | Ala | Ser | Pro | Leu | Thr | Ser | 653 | | | | | | | | | | | | | | | | | | | | | | | | | | |

Fig. 10 (SEQ ID NO: 4)

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|-----|
| | | | | | | | | | | | | | | | | | | | | 10 | | | | | | | | | | | | | | | | | | | | | 20 |
| Gln | Asn | Glu | Asp | Leu | Gly | Pro | Ala | Ser | Pro | Leu | Asp | Ser | Thr | Phe | Tyr | Arg | Ser | Leu | Leu | 20 | | | | | | | | | | | | | | | | | | | | | |
| Glu | Asp | Asp | Asp | Met | Gly | Asp | Leu | Val | Asp | Ala | Glu | Glu | Tyr | Leu | Val | Pro | Gln | Gln | Gly | 40 | | | | | | | | | | | | | | | | | | | | | |
| Phe | Phe | Cys | Pro | Asp | Pro | Ala | Pro | Gly | Ala | Gly | Gly | Met | Val | His | His | Arg | His | Arg | Ser | 60 | | | | | | | | | | | | | | | | | | | | | |
| Ser | Ser | Thr | Arg | Ser | Gly | Gly | Gly | Asp | Leu | Thr | Leu | Gly | Leu | Glu | Pro | Ser | Glu | Glu | Glu | 80 | | | | | | | | | | | | | | | | | | | | | |
| Ala | Pro | Arg | Ser | Pro | Leu | Ala | Pro | Ser | Glu | Gly | Ala | Gly | Ser | Asp | Val | Phe | Asp | Gly | Asp | 100 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | 110 | | | | | | | | | | | | | | | | | | | | | 120 |
| Leu | Gly | Met | Gly | Ala | Ala | Lys | Gly | Leu | Gln | Ser | Leu | Pro | Thr | His | Asp | Pro | Ser | Pro | Leu | 120 | | | | | | | | | | | | | | | | | | | | | |
| Gln | Arg | Tyr | Ser | Glu | Asp | Pro | Thr | Val | Pro | Leu | Pro | Ser | Glu | Thr | Asp | Gly | Tyr | Val | Ala | 140 | | | | | | | | | | | | | | | | | | | | | |
| Pro | Leu | Thr | Cys | Ser | Pro | Gln | Pro | Glu | Tyr | Val | Asn | Gln | Pro | Asp | Val | Arg | Pro | Gln | Pro | 160 | | | | | | | | | | | | | | | | | | | | | |
| Pro | Ser | Pro | Arg | Glu | Gly | Pro | Leu | Pro | Ala | Ala | Arg | Pro | Ala | Gly | Ala | Thr | Leu | Glu | Arg | 180 | | | | | | | | | | | | | | | | | | | | | |
| Pro | Lys | Thr | Leu | Ser | Pro | Gly | Lys | Asn | Gly | Val | Val | Lys | Asp | Val | Phe | Ala | Phe | Gly | Gly | 200 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | 210 | | | | | | | | | | | | | | | | | | | | | 220 |
| Ala | Val | Glu | Asn | Pro | Glu | Tyr | Leu | Thr | Pro | Gln | Gly | Gly | Ala | Ala | Pro | Gln | Pro | His | Pro | 220 | | | | | | | | | | | | | | | | | | | | | |
| Pro | Pro | Ala | Phe | Ser | Pro | Ala | Phe | Asp | Asn | Leu | Tyr | Tyr | Trp | Asp | Gln | Asp | Pro | Pro | Glu | 240 | | | | | | | | | | | | | | | | | | | | | |
| Arg | Gly | Ala | Pro | Pro | Ser | Thr | Phe | Lys | Gly | Thr | Pro | Thr | Ala | Glu | Asn | Pro | Glu | Tyr | Leu | 260 | | | | | | | | | | | | | | | | | | | | | |
| Gly | Leu | Asp | Val | Pro | Val | | | | | | | | | | | | | | | 267 | | | | | | | | | | | | | | | | | | | | | |

Fig. 12 (SEQ ID NO: 6)

| | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | | | | | | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | 20 | | | | | | | | | |
| Met | Glu | Leu | Ala | Ala | Leu | Cys | Arg | Trp | Gly | Leu | Leu | Leu | Ala | Leu | Leu | Pro | Pro | Gly | Ala |
| Ala | Ser | Thr | Gln | Val | Cys | Thr | Gly | Thr | Asp | Met | Lys | Leu | Arg | Leu | Pro | Ala | Ser | Pro | Glu |
| Thr | His | Leu | Asp | Met | Leu | Arg | His | Leu | Tyr | Gln | Gly | Cys | Gln | Val | Val | Gln | Gly | Asn | Leu |
| Glu | Leu | Thr | Tyr | Leu | Pro | Thr | Asn | Ala | Ser | Leu | Ser | Phe | Leu | Gln | Asp | Ile | Gln | Glu | Val |
| Gln | Gly | Tyr | Val | Leu | Ile | Ala | His | Asn | Gln | Val | Arg | Gln | Val | Pro | Leu | Gln | Arg | Leu | Arg |
| 110 | | | | | | | | | | 120 | | | | | | | | | |
| Ile | Val | Arg | Gly | Thr | Gln | Leu | Phe | Glu | Asp | Asn | Tyr | Ala | Leu | Ala | Val | Leu | Asp | Asn | Gly |
| Asp | Pro | Leu | Asn | Asn | Thr | Thr | Pro | Val | Thr | Gly | Ala | Ser | Pro | Gly | Gly | Leu | Arg | Glu | Leu |
| Gln | Leu | Arg | Ser | Leu | Thr | Glu | Ile | Leu | Lys | Gly | Gly | Val | Leu | Ile | Gln | Arg | Asn | Pro | Gln |
| Leu | Cys | Tyr | Gln | Asp | Thr | Ile | Leu | Trp | Lys | Asp | Ile | Phe | His | Lys | Asn | Asn | Gln | Leu | Ala |
| Leu | Thr | Leu | Ile | Asp | Thr | Asn | Arg | Ser | Arg | Ala | Cys | His | Pro | Cys | Ser | Pro | Met | Cys | Lys |
| 210 | | | | | | | | | | 220 | | | | | | | | | |
| Gly | Ser | Arg | Cys | Trp | Gly | Glu | Ser | Ser | Glu | Asp | Cys | Gln | Ser | Leu | Thr | Arg | Thr | Val | Cys |
| Ala | Gly | Gly | Cys | Ala | Arg | Cys | Lys | Gly | Pro | Leu | Pro | Thr | Asp | Cys | Cys | His | Glu | Gln | Cys |
| Ala | Ala | Gly | Cys | Thr | Gly | Pro | Lys | His | Ser | Asp | Cys | Leu | Ala | Cys | Leu | His | Phe | Asn | His |
| Ser | Gly | Ile | Cys | Glu | Leu | His | Cys | Pro | Ala | Leu | Val | Thr | Tyr | Asn | Thr | Asp | Thr | Phe | Glu |
| Ser | Met | Pro | Asn | Pro | Glu | Gly | Arg | Tyr | Thr | Phe | Gly | Ala | Ser | Cys | Val | Thr | Ala | Cys | Pro |
| 310 | | | | | | | | | | 320 | | | | | | | | | |
| Tyr | Asn | Tyr | Leu | Ser | Thr | Asp | Val | Gly | Ser | Cys | Thr | Leu | Val | Cys | Pro | Leu | His | Asn | Gln |
| Glu | Val | Thr | Ala | Glu | Asp | Gly | Thr | Gln | Arg | Cys | Glu | Lys | Cys | Ser | Lys | Pro | Cys | Ala | Arg |
| Val | Cys | Tyr | Gly | Leu | Gly | Met | Glu | His | Leu | Arg | Glu | Val | Arg | Ala | Val | Thr | Ser | Ala | Asn |
| Ile | Gln | Glu | Phe | Ala | Gly | Cys | Lys | Lys | Ile | Phe | Gly | Ser | Leu | Ala | Phe | Leu | Pro | Glu | Ser |
| Phe | Asp | Gly | Asp | Pro | Ala | Ser | Asn | Thr | Ala | Pro | Leu | Gln | Pro | Glu | Gln | Leu | Gln | Val | Phe |
| 410 | | | | | | | | | | 420 | | | | | | | | | |
| Glu | Thr | Leu | Glu | Glu | Ile | Thr | Gly | Tyr | Leu | Tyr | Ile | Ser | Ala | Trp | Pro | Asp | Ser | Leu | Pro |
| Asp | Leu | Ser | Val | Phe | Gln | Asn | Leu | Gln | Val | Ile | Arg | Gly | Arg | Ile | Leu | His | Asn | Gly | Ala |
| Tyr | Ser | Leu | Thr | Leu | Gln | Gly | Leu | Gly | Ile | Ser | Trp | Leu | Gly | Leu | Arg | Ser | Leu | Arg | Glu |
| Leu | Gly | Ser | Gly | Leu | Ala | Leu | Ile | His | His | Asn | Thr | His | Leu | Cys | Phe | Val | His | Thr | Val |
| Pro | Trp | Asp | Gln | Leu | Phe | Arg | Asn | Pro | His | Gln | Ala | Leu | Leu | His | Thr | Ala | Asn | Arg | Pro |
| 510 | | | | | | | | | | 520 | | | | | | | | | |
| Glu | Asp | Glu | Cys | Val | Gly | Glu | Gly | Leu | Ala | Cys | His | Gln | Leu | Cys | Ala | Arg | Gly | His | Cys |
| Trp | Gly | Pro | Gly | Pro | Thr | Gln | Cys | Val | Asn | Cys | Ser | Gln | Phe | Leu | Arg | Gly | Gln | Glu | Cys |
| Val | Glu | Glu | Cys | Arg | Val | Leu | Gln | Gly | Leu | Pro | Arg | Glu | Tyr | Val | Asn | Ala | Arg | His | Cys |
| Leu | Pro | Cys | His | Pro | Glu | Cys | Gln | Pro | Gln | Asn | Gly | Ser | Val | Thr | Cys | Phe | Gly | Pro | Glu |
| Ala | Asp | Gln | Cys | Val | Ala | Cys | Ala | His | Tyr | Lys | Asp | Pro | Pro | Phe | Cys | Val | Ala | Arg | Cys |

Fig. 12 (SEQ ID NO: 6)

| | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 610 | | | | | | | | | | 620 | | | | | | | | | | |
| Pro | Ser | Gly | Val | Lys | Pro | Asp | Leu | Ser | Tyr | Met | Pro | Ile | Trp | Lys | Phe | Pro | Asp | Glu | Glu | 620 |
| Gly | Ala | Cys | Gln | Pro | Cys | Pro | Ile | Asn | Cys | Thr | His | Ser | Cys | Val | Asp | Leu | Asp | Asp | Lys | 640 |
| Gly | Cys | Pro | Ala | Glu | Gln | Arg | Ala | Ser | Pro | Leu | Thr | Ser | Gln | Asn | Glu | Asp | Leu | Gly | Pro | 660 |
| Ala | Ser | Pro | Leu | Asp | Ser | Thr | Phe | Tyr | Arg | Ser | Leu | Leu | Glu | Asp | Asp | Asp | Met | Gly | Asp | 680 |
| Leu | Val | Asp | Ala | Glu | Glu | Tyr | Leu | Val | Pro | Gln | Gln | Gly | Phe | Phe | Cys | Pro | Asp | Pro | Ala | 700 |
| 710 | | | | | | | | | | 720 | | | | | | | | | | |
| Pro | Gly | Ala | Gly | Gly | Met | Val | His | His | Arg | His | Arg | Ser | Ser | Ser | Thr | Arg | Ser | Gly | Gly | 720 |
| Gly | Asp | Leu | Thr | Leu | Gly | Leu | Glu | Pro | Ser | Glu | Glu | Glu | Ala | Pro | Arg | Ser | Pro | Leu | Ala | 740 |
| Pro | Ser | Glu | Gly | Ala | Gly | Ser | Asp | Val | Phe | Asp | Gly | Asp | Leu | Gly | Met | Gly | Ala | Ala | Lys | 760 |
| Gly | Leu | Gln | Ser | Leu | Pro | Thr | His | Asp | Pro | Ser | Pro | Leu | Gln | Arg | Tyr | Ser | Glu | Asp | Pro | 780 |
| Thr | Val | Pro | Leu | Pro | Ser | Glu | Thr | Asp | Gly | Tyr | Val | Ala | Pro | Leu | Thr | Cys | Ser | Pro | Gln | 800 |
| 810 | | | | | | | | | | 820 | | | | | | | | | | |
| Pro | Glu | Tyr | Val | Asn | Gln | Pro | Asp | Val | Arg | Pro | Gln | Pro | Pro | Ser | Pro | Arg | Glu | Gly | Pro | 820 |
| Leu | Pro | Ala | Ala | Arg | Pro | Ala | Gly | Ala | Thr | Leu | Glu | Arg | Pro | Lys | Thr | Leu | Ser | Pro | Gly | 840 |
| Lys | Asn | Gly | Val | Val | Lys | Asp | Val | Phe | Ala | Phe | Gly | Gly | Ala | Val | Glu | Asn | Pro | Glu | Tyr | 860 |
| Leu | Thr | Pro | Gln | Gly | Gly | Ala | Ala | Pro | Gln | Pro | His | Pro | Pro | Pro | Ala | Phe | Ser | Pro | Ala | 880 |
| Phe | Asp | Asn | Leu | Tyr | Tyr | Trp | Asp | Gln | Asp | Pro | Pro | Glu | Arg | Gly | Ala | Pro | Pro | Ser | Thr | 900 |
| 910 | | | | | | | | | | 920 | | | | | | | | | | |
| Phe | Lys | Gly | Thr | Pro | Thr | Ala | Glu | Asn | Pro | Glu | Tyr | Leu | Gly | Leu | Asp | Val | Pro | Val | • | 920 |

• •

Fig. 14 (SEQ ID NO: 8)

| | | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 10 | | | | | | | | | | 20 | | | | | | | | | | |
| Met | Glu | Leu | Ala | Ala | Trp | Cys | Arg | Trp | Gly | Phe | Leu | Leu | Ala | Leu | Leu | Pro | Pro | Gly | Ile | 20 |
| Ala | Gly | Thr | Gln | Val | Cys | Thr | Gly | Thr | Asp | Met | Lys | Leu | Arg | Leu | Pro | Ala | Ser | Pro | Glu | 40 |
| Thr | His | Leu | Asp | Met | Leu | Arg | His | Leu | Tyr | Gln | Gly | Cys | Gln | Val | Val | Gln | Gly | Asn | Leu | 60 |
| Glu | Leu | Thr | Tyr | Val | Pro | Ala | Asn | Ala | Ser | Leu | Ser | Phe | Leu | Gln | Asp | Ile | Gln | Glu | Val | 80 |
| Gln | Gly | Tyr | Met | Leu | Ile | Ala | His | Asn | Gln | Val | Lys | Arg | Val | Pro | Leu | Gln | Arg | Leu | Arg | 100 |
| 110 | | | | | | | | | | 120 | | | | | | | | | | |
| Ile | Val | Arg | Gly | Thr | Gln | Leu | Phe | Glu | Asp | Lys | Tyr | Ala | Leu | Ala | Val | Leu | Asp | Asn | Arg | 120 |
| Asp | Pro | Gln | Asp | Asn | Val | Ala | Ala | Ser | Thr | Pro | Gly | Arg | Thr | Pro | Glu | Gly | Leu | Arg | Glu | 140 |
| Leu | Gln | Leu | Arg | Ser | Leu | Thr | Glu | Ile | Leu | Lys | Gly | Gly | Val | Leu | Ile | Arg | Gly | Asn | Pro | 160 |
| Gln | Leu | Cys | Tyr | Gln | Asp | Met | Val | Leu | Trp | Lys | Asp | Val | Phe | Arg | Lys | Asn | Asn | Gln | Leu | 180 |
| Ala | Pro | Val | Asp | Ile | Asp | Thr | Asn | Arg | Ser | Arg | Ala | Cys | Pro | Pro | Cys | Ala | Pro | Ala | Cys | 200 |
| 210 | | | | | | | | | | 220 | | | | | | | | | | |
| Lys | Asp | Asn | His | Cys | Trp | Gly | Glu | Ser | Pro | Glu | Asp | Cys | Gln | Ile | Leu | Thr | Gly | Thr | Ile | 220 |
| Cys | Thr | Ser | Gly | Cys | Ala | Arg | Cys | Lys | Gly | Arg | Leu | Pro | Thr | Asp | Cys | Cys | His | Glu | Gln | 240 |
| Cys | Ala | Ala | Gly | Cys | Thr | Gly | Pro | Lys | His | Ser | Asp | Cys | Leu | Ala | Cys | Leu | His | Phe | Asn | 260 |
| His | Ser | Gly | Ile | Cys | Glu | Leu | His | Cys | Pro | Ala | Leu | Val | Thr | Tyr | Asn | Thr | Asp | Thr | Phe | 280 |
| Glu | Ser | Met | His | Asn | Pro | Glu | Gly | Arg | Tyr | Thr | Phe | Gly | Ala | Ser | Cys | Val | Thr | Thr | Cys | 300 |
| 310 | | | | | | | | | | 320 | | | | | | | | | | |
| Pro | Tyr | Asn | Tyr | Leu | Ser | Thr | Glu | Val | Gly | Ser | Cys | Thr | Leu | Val | Cys | Pro | Pro | Asn | Asn | 320 |
| Gln | Glu | Val | Thr | Ala | Glu | Asp | Gly | Thr | Gln | Arg | Cys | Glu | Lys | Cys | Ser | Lys | Pro | Cys | Ala | 340 |
| Arg | Val | Cys | Tyr | Gly | Leu | Gly | Met | Glu | His | Leu | Arg | Gly | Ala | Arg | Ala | Ile | Thr | Ser | Asp | 360 |
| Asn | Val | Gln | Glu | Phe | Asp | Gly | Cys | Lys | Lys | Ile | Phe | Gly | Ser | Leu | Ala | Phe | Leu | Pro | Glu | 380 |
| Ser | Phe | Asp | Gly | Asp | Pro | Ser | Ser | Gly | Ile | Ala | Pro | Leu | Arg | Pro | Glu | Gln | Leu | Gln | Val | 400 |
| 410 | | | | | | | | | | 420 | | | | | | | | | | |
| Phe | Glu | Thr | Leu | Glu | Glu | Ile | Thr | Gly | Tyr | Leu | Tyr | Ile | Ser | Ala | Trp | Pro | Asp | Ser | Leu | 420 |
| Arg | Asp | Leu | Ser | Val | Phe | Gln | Asn | Leu | Arg | Ile | Ile | Arg | Gly | Arg | Ile | Leu | His | Asp | Gly | 440 |
| Ala | Tyr | Ser | Leu | Thr | Leu | Gln | Gly | Leu | Gly | Ile | His | Ser | Leu | Gly | Leu | Arg | Ser | Leu | Arg | 460 |
| Glu | Leu | Gly | Ser | Gly | Leu | Ala | Leu | Ile | His | Arg | Asn | Ala | His | Leu | Cys | Phe | Val | His | Thr | 480 |
| Val | Pro | Trp | Asp | Gln | Leu | Phe | Arg | Asn | Pro | His | Gln | Ala | Leu | Leu | His | Ser | Gly | Asn | Arg | 500 |
| 510 | | | | | | | | | | 520 | | | | | | | | | | |
| Pro | Glu | Glu | Asp | Cys | Gly | Leu | Glu | Gly | Leu | Val | Cys | Asn | Ser | Leu | Cys | Ala | His | Gly | His | 520 |
| Cys | Trp | Gly | Pro | Gly | Pro | Thr | Gln | Cys | Val | Asn | Cys | Ser | His | Phe | Leu | Arg | Gly | Gln | Glu | 540 |
| Cys | Val | Glu | Glu | Cys | Arg | Val | Trp | Lys | Gly | Leu | Pro | Arg | Glu | Tyr | Val | Ser | Asp | Lys | Arg | 560 |
| Cys | Leu | Pro | Cys | His | Pro | Glu | Cys | Gln | Pro | Gln | Asn | Ser | Ser | Glu | Thr | Cys | Phe | Gly | Ser | 580 |
| Glu | Ala | Asp | Gln | Cys | Ala | Ala | Cys | Ala | His | Tyr | Lys | Asp | Ser | Ser | Ser | Cys | Val | Ala | Arg | 600 |
| 610 | | | | | | | | | | 620 | | | | | | | | | | |
| Cys | Pro | Ser | Gly | Val | Lys | Pro | Asp | Leu | Ser | Tyr | Met | Pro | Ile | Trp | Lys | Tyr | Pro | Asp | Glu | 620 |
| Glu | Gly | Ile | Cys | Gln | Pro | Cys | Pro | Ile | Asn | Cys | Thr | His | Ser | Cys | Val | Asp | Leu | Asp | Glu | 640 |
| Arg | Gly | Cys | Pro | Ala | Glu | Gln | Arg | Ala | Ser | Pro | Val | Thr | Phe | | | | | | | 654 |

Fig. 15 (SEQ ID NO: 9)

| | |
|-----------------------------------------------------------------|-----|
| ATG GAG CTG GCG GCC TTG TGC CGC TGG GGG CTC CTC CTC GCC CTC TTG | 48 |
| Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu | |
| 1 5 10 15 | |
| CCC CCC GGA GCC GCG AGC ACC CAA GTG TGC ACC GGC ACA GAC ATG AAG | 96 |
| Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys | |
| 20 25 30 | |
| CTG CGG CTC CCT GCC AGT CCC GAG ACC CAC CTG GAC ATG CTC CGC CAC | 144 |
| Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His | |
| 35 40 45 | |
| CTC TAC CAG GGC TGC CAG GTG GTG CAG GGA AAC CTG GAA CTC ACC TAC | 192 |
| Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr | |
| 50 55 60 | |
| CTG CCC ACC AAT GCC AGC CTG TCC TTC CTG CAG GAT ATC CAG GAG GTG | 240 |
| Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val | |
| 65 70 75 80 | |
| CAG GGC TAC GTG CTC ATC GCT CAC AAC CAA GTG AGG CAG GTC CCA CTG | 288 |
| Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu | |
| 85 90 95 | |
| CAG AGG CTG CGG ATT GTG CGA GGC ACC CAG CTC TTT GAG GAC AAC TAT | 336 |
| Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr | |
| 100 105 110 | |
| GCC CTG GCC GTG CTA GAC AAT GGA GAC CCG CTG AAC AAT ACC ACC CCT | 384 |
| Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro | |
| 115 120 125 | |

Fig. 15 (SEQ ID NO: 9)

| | |
|-----------------------------------------------------------------|-----|
| GTC ACA GGG GCC TCC CCA GGA GGC CTG CGG GAG CTG CAG CTT CGA AGC | 432 |
| Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser | |
| 130 135 140 | |
| CTC ACA GAG ATC TTG AAA GGA GGG GTC TTG ATC CAG CGG AAC CCC CAG | 480 |
| Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln | |
| 145 150 155 160 | |
| CTC TGC TAC CAG GAC ACG ATT TTG TGG AAG GAC ATC TTC CAC AAG AAC | 528 |
| Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn | |
| 165 170 175 | |
| AAC CAG CTG GCT CTC ACA CTG ATA GAC ACC AAC CGC TCT CGG GCC TGC | 576 |
| Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys | |
| 180 185 190 | |
| CAC CCC TGT TCT CCG ATG TGT AAG GGC TCC CGC TGC TGG GGA GAG AGT | 624 |
| His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser | |
| 195 200 205 | |
| TCT GAG GAT TGT CAG AGC CTG ACG CGC ACT GTC TGT GCC GGT GGC TGT | 672 |
| Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys | |
| 210 215 220 | |
| GCC CGC TGC AAG GGG CCA CTG CCC ACT GAC TGC TGC CAT GAG CAG TGT | 720 |
| Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys | |
| 225 230 235 240 | |
| GCT GCC GGC TGC ACG GGC CCC AAG CAC TCT GAC TGC CTG GCC TGC CTC | 768 |
| Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu | |
| 245 250 255 | |
| CAC TTC AAC CAC AGT GGC ATC TGT GAG CTG CAC TGC CCA GCC CTG GTC | 816 |
| His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val | |
| 260 265 270 | |
| ACC TAC AAC ACA GAC ACG TTT GAG TCC ATG CCC AAT CCC GAG GGC CGG | 864 |
| Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg | |
| 275 280 285 | |
| TAT ACA TTC GGC GCC AGC TGT GTG ACT GCC TGT CCC TAC AAC TAC CTT | 912 |
| Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu | |
| 290 295 300 | |

| | 1970 | 1971 | 1972 | 1973 | 1974 | 1975 | 1976 | 1977 | 1978 | 1979 | 1980 | 1981 | 1982 | 1983 | 1984 | 1985 | 1986 | 1987 | 1988 | 1989 | 1990 | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 | 2024 | 2025 | 2026 | 2027 | 2028 | 2029 | 2030 | 2031 | 2032 | 2033 | 2034 | 2035 | 2036 | 2037 | 2038 | 2039 | 2040 | 2041 | 2042 | 2043 | 2044 | 2045 | 2046 | 2047 | 2048 | 2049 | 2050 | 2051 | 2052 | 2053 | 2054 | 2055 | 2056 | 2057 | 2058 | 2059 | 2060 | 2061 | 2062 | 2063 | 2064 | 2065 | 2066 | 2067 | 2068 | 2069 | 2070 | 2071 | 2072 | 2073 | 2074 | 2075 | 2076 | 2077 | 2078 | 2079 | 2080 | 2081 | 2082 | 2083 | 2084 | 2085 | 2086 | 2087 | 2088 | 2089 | 2090 | 2091 | 2092 | 2093 | 2094 | 2095 | 2096 | 2097 | 2098 | 2099 | 2100 | 2101 | 2102 | 2103 | 2104 | 2105 | 2106 | 2107 | 2108 | 2109 | 2110 | 2111 | 2112 | 2113 | 2114 | 2115 | 2116 | 2117 | 2118 | 2119 | 2120 | 2121 | 2122 | 2123 | 2124 | 2125 | 2126 | 2127 | 2128 | 2129 | 2130 | 2131 | 2132 | 2133 | 2134 | 2135 | 2136 | 2137 | 2138 | 2139 | 2140 | 2141 | 2142 | 2143 | 2144 | 2145 | 2146 | 2147 | 2148 | 2149 | 2150 | 2151 | 2152 | 2153 | 2154 | 2155 | 2156 | 2157 | 2158 | 2159 | 2160 | 2161 | 2162 | 2163 | 2164 | 2165 | 2166 | 2167 | 2168 | 2169 | 2170 | 2171 | 2172 | 2173 | 2174 | 2175 | 2176 | 2177 | 2178 | 2179 | 2180 | 2181 | 2182 | 2183 | 2184 | 2185 | 2186 | 2187 | 2188 | 2189 | 2190 | 2191 | 2192 | 2193 | 2194 | 2195 | 2196 | 2197 | 2198 | 2199 | 2200 | 2201 | 2202 | 2203 | 2204 | 2205 | 2206 | 2207 | 2208 | 2209 | 2210 | 2211 | 2212 | 2213 | 2214 | 2215 | 2216 | 2217 | 2218 | 2219 | 2220 | 2221 | 2222 | 2223 | 2224 | 2225 | 2226 | 2227 | 2228 | 2229 | 2230 | 2231 | 2232 | 2233 | 2234 | 2235 | 2236 | 2237 | 2238 | 2239 | 2240 | 2241 | 2242 | 2243 | 2244 | 2245 | 2246 | 2247 | 2248 | 2249 | 2250 | 2251 | 2252 | 2253 | 2254 | 2255 | 2256 | 2257 | 2258 | 2259 | 2260 | 2261 | 2262 | 2263 | 2264 | 2265 | 2266 | 2267 | 2268 | 2269 | 2270 | 2271 | 2272 | 2273 | 2274 | 2275 | 2276 | 2277 | 2278 | 2279 | 2280 | 2281 | 2282 | 2283 | 2284 | 2285 | 2286 | 2287 | 2288 | 2289 | 2290 | 2291 | 2292 | 2293 | 2294 | 2295 | 2296 | 2297 | 2298 | 2299 | 2300 | 2301 | 2302 | 2303 | 2304 | 2305 | 2306 | 2307 | 2308 | 2309 | 2310 | 2311 | 2312 | 2313 | 2314 | 2315 | 2316 | 2317 | 2318 | 2319 | 2320 | 2321 | 2322 | 2323 | 2324 | 2325 | 2326 | 2327 | 2328 | 2329 | 2330 | 2331 | 2332 | 2333 | 2334 | 2335 | 2336 | 2337 | 2338 | 2339 | 2340 | 2341 | 2342 | 2343 | 2344 | 2345 | 2346 | 2347 | 2348 | 2349 | 2350 | 2351 | 2352 | 2353 | 2354 | 2355 | 2356 | 2357 | 2358 | 2359 | 2360 | 2361 | 2362 | 2363 | 2364 | 2365 | 2366 | 2367 | 2368 | 2369 | 2370 | 2371 | 2372 | 2373 | 2374 | 2375 | 2376 | 2377 | 2378 | 2379 | 2380 | 2381 | 2382 | 2383 | 2384 | 2385 | 2386 | 2387 | 2388 | 2389 | 2390 | 2391 | 2392 | 2393 | 2394 | 2395 | 2396 | 2397 | 2398 | 2399 | 2400 | 2401 | 2402 | 2403 | 2404 | 2405 | 2406 | 2407 | 2408 | 2409 | 2410 | 2411 | 2412 | 2413 | 2414 | 2415 | 2416 | 2417 | 2418 | 2419 | 2420 | 2421 | 2422 | 2 |
|--|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|---|
|--|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|---|

| | |
|-----------------------------------------------------------------|------|
| TCT ACG GAC GTG GGA TCC TGC ACC CTC GTC TGC CCC CTG CAC AAC CAA | 960 |
| Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln | |
| 305 310 315 320 | |
| GAG GTG ACA GCA GAG GAT GGA ACA CAG CGG TGT GAG AAG TGC AGC AAG | 1008 |
| Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys | |
| 325 330 335 | |
| CCC TGT GCC CGA GTG TGC TAT GGT CTG GGC ATG GAG CAC TTG CGA GAG | 1056 |
| Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu | |
| 340 345 350 | |
| GTG AGG GCA GTT ACC AGT GCC AAT ATC CAG GAG TTT GCT GGC TGC AAG | 1104 |
| Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys | |
| 355 360 365 | |
| AAG ATC TTT GGG AGC CTG GCA TTT CTG CCG GAG AGC TTT GAT GGG GAC | 1152 |
| Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp | |
| 370 375 380 | |
| CCA GCC TCC AAC ACT GCC CCG CTC CAG CCA GAG CAG CTC CAA GTG TTT | 1200 |
| Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe | |
| 385 390 395 400 | |
| GAG ACT CTG GAA GAG ATC ACA GGT TAC CTA TAC ATC TCA GCA TGG CCG | 1248 |
| Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro | |
| 405 410 415 | |
| GAC AGC CTG CCT GAC CTC AGC GTC TTC CAG AAC CTG CAA GTA ATC CGG | 1296 |
| Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg | |
| 420 425 430 | |
| GGA CGA ATT CTG CAC AAT GGC GCC TAC TCG CTG ACC CTG CAA GGG CTG | 1344 |
| Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu | |
| 435 440 445 | |
| GGC ATC AGC TGG CTG GGG CTG CGC TCA CTG AGG GAA CTG GGC AGT GGA | 1392 |
| Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly | |
| 450 455 460 | |
| CTG GCC CTC ATC CAC CAT AAC ACC CAC CTC TGC TTC GTG CAC ACG GTG | 1440 |
| Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val | |
| 465 470 475 480 | |

Fig. 15 (SEQ ID NO: 9)

| | |
|-----------------------------------------------------------------|------|
| CCC TGG GAC CAG CTC TTT CGG AAC CCG CAC CAA GCT CTG CTC CAC ACT | 1488 |
| Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr | |
| 485 490 495 | |
| GCC AAC CGG CCA GAG GAC GAG TGT GTG GGC GAG GGC CTG GCC TGC CAC | 1536 |
| Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His | |
| 500 505 510 | |
| CAG CTG TGC GCC CGA GGG CAC TGC TGG GGT CCA GGG CCC ACC CAG TGT | 1584 |
| Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys | |
| 515 520 525 | |
| GTC AAC TGC AGC CAG TTC CTT CGG GGC CAG GAG TGC GTG GAG GAA TGC | 1632 |
| Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys | |
| 530 535 540 | |
| CGA GTA CTG CAG GGG CTC CCC AGG GAG TAT GTG AAT GCC AGG CAC TGT | 1680 |
| Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys | |
| 545 550 555 560 | |
| TTG CCG TGC CAC CCT GAG TGT CAG CCC CAG AAT GGC TCA GTG ACC TGT | 1728 |
| Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys | |
| 565 570 575 | |
| TTT GGA CCG GAG GCT GAC CAG TGT GTG GCC TGT GCC CAC TAT AAG GAC | 1776 |
| Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp | |
| 580 585 590 | |
| CCT CCC TTC TGC GTG GCC CGC TGC CCC AGC GGT GTG AAA CCT GAC CTC | 1824 |
| Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu | |
| 595 600 605 | |
| TCC TAC ATG CCC ATC TGG AAG TTT CCA GAT GAG GAG GGC GCA TGC CAG | 1872 |
| Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln | |
| 610 615 620 | |
| CCT TGC CCC ATC AAC TGC ACC CAC TCC TGT GTG GAC CTG GAT GAC AAG | 1920 |
| Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys | |
| 625 630 635 640 | |
| GGC TGC CCC GCC GAG CAG AGA GCC AGC CCT CTG ACG TCC ATC ATC TCT | 1968 |
| Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Ile Ser | |
| 645 650 655 | |
| GCG GTG GTT GGC ATT CTG CTG GTC GTG GTC TTG GGG GTG GTC TTT GGG | 2016 |
| Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly | |
| 660 665 670 | |

Fig. 15 (SEQ ID NO: 9)

| | |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| ATC CTC ATC AAG CGA CGG CAG CAG AAG ATC CGG AAG TAC ACG ATG CGG Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg 675 680 685 | 2064 |
| AGA CTG CTG CAG GAA ACG GAG CTG GTG GAG CCG CTG ACA CCT AGC GGA Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly 690 695 700 | 2112 |
| GCG ATG CCC AAC CAG GCG CAG ATG CGG ATC CTG AAA GAG ACG GAG CTG Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu 705 710 715 720 | 2160 |
| AGG AAG GTG AAG GTG CTT GGA TCT GGC GCT TTT GGC ACA GTC TAC AAG Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys 725 730 735 | 2208 |
| GGC ATC TGG ATC CCT GAT GGG GAG AAT GTG AAA ATT CCA GTG GCC ATC Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile 740 745 750 | 2256 |
| AAA GTG TTG AGG GAA AAC ACA TCC CCC AAA GCC AAC AAA GAA ATC TTA Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu 755 760 765 | 2304 |
| GAC GAA GCA TAC GTG ATG GCT GGT GTG GGC TCC CCA TAT GTC TCC CGC Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg 770 775 780 | 2352 |
| CTT CTG GGC ATC TGC CTG ACA TCC ACG GTG CAG CTG GTG ACA CAG CTT Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu 785 790 795 800 | 2400 |
| ATG CCC TAT GGC TGC CTC TTA GAC CAT GTC CGG GAA AAC CGC GGA CGC Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg 805 810 815 | 2448 |
| CTG GGC TCC CAG GAC CTG CTG AAC TGG TGT ATG CAG ATT GCC AAG GGG Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly 820 825 830 | 2496 |
| ATG AGC TAC CTG GAG GAT GTG CGG CTC GTA CAC AGG GAC TTG GCC GCT Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala 835 840 845 | 2544 |

Fig. 15 (SEQ ID NO: 9)

| | |
|-----------------------------------------------------------------|------|
| CGG AAC GTG CTG GTC AAG AGT CCC AAC CAT GTC AAA ATT ACA GAC TTC | 2592 |
| Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe | |
| 850 855 860 | |
| GGG CTG GCT CGG CTG CTG GAC ATT GAC GAG ACA GAG TAC CAT GCA GAT | 2640 |
| Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp | |
| 865 870 875 880 | |
| GGG GGC AAG GTG CCC ATC AAG TGG ATG GCG CTG GAG TCC ATT CTC CGC | 2688 |
| Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg | |
| 885 890 895 | |
| CGG CGG TTC ACC CAC CAG AGT GAT GTG TGG AGT TAT GGT GTG ACT GTG | 2736 |
| Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val | |
| 900 905 910 | |
| TGG GAG CTG ATG ACT TTT GGG GCC AAA CCT TAC GAT GGG ATC CCA GCC | 2784 |
| Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala | |
| 915 920 925 | |
| CGG GAG ATC CCT GAC CTG CTG GAA AAG GGG GAG CGG CTG CCC CAG CCC | 2832 |
| Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro | |
| 930 935 940 | |
| CCC ATC TGC ACC ATT GAT GTC TAC ATG ATC ATG GTC AAA TGT TGG ATG | 2880 |
| Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met | |
| 945 950 955 960 | |
| ATT GAC TCT GAA TGT CGG CCA AGA TTC CGG GAG TTG GTG TCT GAA TTC | 2928 |
| Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe | |
| 965 970 975 | |
| TCC CGC ATG GCC AGG GAC CCC CAG CGC TTT GTG GTC ATC CAG AAT GAG | 2976 |
| Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu | |
| 980 985 990 | |
| GAC TTG GGC CCA GCC AGT CCC TTG GAC AGC ACC TTC TAC CGC TCA CTG | 3024 |
| Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu | |
| 995 1000 1005 | |
| CTG GAG GAC GAT GAC ATG GGG GAC CTG GTG GAT GCT GAG GAG TAT CTG | 3072 |
| Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu | |
| 1010 1015 1020 | |

Fig. 15 (SEQ ID NO: 9)

| | |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| GTA CCC CAG CAG GGC TTC TTC TGT CCA GAC CCT GCC CCG GGC GCT GGG Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly 1025 1030 1035 1040 | 3120 |
| GGC ATG GTC CAC CAC AGG CAC CGC AGC TCA TCT ACC AGG AGT GGC GGT Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly 1045 1050 1055 | 3168 |
| GGG GAC CTG ACA CTA GGG CTG GAG CCC TCT GAA GAG GAG GCC CCC AGG Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg 1060 1065 1070 | 3216 |
| TCT CCA CTG GCA CCC TCC GAA GGG GCT GGC TCC GAT GTA TTT GAT GGT Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly 1075 1080 1085 | 3264 |
| GAC CTG GGA ATG GGG GCA GCC AAG GGG CTG CAA AGC CTC CCC ACA CAT Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His 1090 1095 1100 | 3312 |
| GAC CCC AGC CCT CTA CAG CGG TAC AGT GAG GAC CCC ACA GTA CCC CTG Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu 1105 1110 1115 1120 | 3360 |
| CCC TCT GAG ACT GAT GGC TAC GTT GCC CCC CTG ACC TGC AGC CCC CAG Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln 1125 1130 1135 | 3408 |
| CCT GAA TAT GTG AAC CAG CCA GAT GTT CGG CCC CAG CCC CCT TCG CCC Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro 1140 1145 1150 | 3456 |
| CGA GAG GGC CCT CTG CCT GCT GCC CGA CCT GCT GGT GCC ACT CTG GAA Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu 1155 1160 1165 | 3504 |
| AGG CCC AAG ACT CTC TCC CCA GGG AAG AAT GGG GTC GTC AAA GAC GTT Arg Pro Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val 1170 1175 1180 | 3552 |
| TTT GCC TTT GGG GGT GCC GTG GAG AAC CCC GAG TAC TTG ACA CCC CAG Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln 1185 1190 1195 1200 | 3600 |

11
 12
 13
 14
 15
 16
 17
 18
 19
 20
 21
 22
 23
 24
 25
 26
 27
 28
 29
 30
 31
 32
 33
 34
 35
 36
 37
 38
 39
 40
 41
 42
 43
 44
 45
 46
 47
 48
 49
 50
 51
 52
 53
 54
 55
 56
 57
 58
 59
 60
 61
 62
 63
 64
 65
 66
 67
 68
 69
 70
 71
 72
 73
 74
 75
 76
 77
 78
 79
 80
 81
 82
 83
 84
 85
 86
 87
 88
 89
 90
 91
 92
 93
 94
 95
 96
 97
 98
 99
 100
 101
 102
 103
 104
 105
 106
 107
 108
 109
 110
 111
 112
 113
 114
 115
 116
 117
 118
 119
 120
 121
 122
 123
 124
 125
 126
 127
 128
 129
 130
 131
 132
 133
 134
 135
 136
 137
 138
 139
 140
 141
 142
 143
 144
 145
 146
 147
 148
 149
 150
 151
 152
 153
 154
 155
 156
 157
 158
 159
 160
 161
 162
 163
 164
 165
 166
 167
 168
 169
 170
 171
 172
 173
 174
 175
 176
 177
 178
 179
 180
 181
 182
 183
 184
 185
 186
 187
 188
 189
 190
 191
 192
 193
 194
 195
 196
 197
 198
 199
 200
 201
 202
 203
 204
 205
 206
 207
 208
 209
 210
 211
 212
 213
 214
 215
 216
 217
 218
 219
 220
 221
 222
 223
 224
 225
 226
 227
 228
 229
 230
 231
 232
 233
 234
 235
 236
 237
 238
 239
 240
 241
 242
 243
 244
 245
 246
 247
 248
 249
 250
 251
 252
 253
 254
 255
 256
 257
 258
 259
 260
 261
 262
 263
 264
 265
 266
 267
 268
 269
 270
 271
 272
 273
 274
 275
 276
 277
 278
 279
 280
 281
 282
 283
 284
 285
 286
 287
 288
 289
 290
 291
 292
 293
 294
 295
 296
 297
 298
 299
 300
 301
 302
 303
 304
 305
 306
 307
 308
 309
 310
 311
 312
 313
 314
 315
 316
 317
 318
 319
 320
 321
 322
 323
 324
 325
 326
 327
 328
 329
 330
 331
 332
 333
 334
 335
 336
 337
 338
 339
 340
 341
 342
 343
 344
 345
 346
 347
 348
 349
 350
 351
 352
 353
 354
 355
 356
 357
 358
 359
 360
 361
 362
 363
 364
 365
 366
 367
 368
 369
 370
 371
 372
 373
 374
 375
 376
 377
 378
 379
 380
 381
 382
 383
 384
 385
 386
 387
 388
 389
 390
 391
 392
 393
 394
 395
 396
 397
 398
 399
 400
 401
 402
 403
 404
 405
 406
 407
 408
 409
 410
 411
 412
 413
 414
 415
 416
 417
 418
 419
 420
 421
 422
 423
 424
 425
 426
 427
 428
 429
 430
 431
 432
 433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456
 457
 458
 459
 460
 461
 462
 463
 464
 465
 466
 467
 468
 469
 470
 471
 472
 473
 474
 475
 476
 477
 478
 479
 480
 481
 482
 483
 484
 485
 486
 487
 488
 489
 490
 491
 492
 493
 494
 495
 496
 497
 498
 499
 500
 501
 502
 503
 504
 505
 506
 507
 508
 509
 510
 511
 512
 513
 514
 515
 516
 517
 518
 519
 520
 521
 522
 523
 524
 525
 526
 527
 528
 529
 530
 531
 532
 533

Fig. 16 (SEQ ID NO: 10)

```

2401 ggcattccaga gttccagctgg ccacacaggt tatggcctac ggcctgcttc tggaccatgt
2411 ccgaggaacac cggaggtggc ccagctggcc ggacctggcc aacggggttg tccagattgc
2511 ccgaggggttg agctgctggg aggaagctgg gcttggctac agggacatgg ctgcccggga
2561 tgggctagtc caggagctggg accaggtgga gattacagat tccgggctgg ctgggctggc
2641 ggcgattgat gaggacggagt acaatgcaaa tggggggcaag gtcgccattc gattggatggc
2701 attggaatct attcctgggg gtcgggttcac ccattcggagt gatgtggtga gcatggagt
2761 gactggtgag gggctgattg atttggggc caaaccttac gatgggaatc caggccggga
2801 gatccctgat tggctgggga agggggaacg cctacctcag cctcgaatcc gacccattga
2881 ggtctacatg attatgggtc agtctgggtt gattgaatct gaatgctggc cggagattccg
2941 ggggttgggt ccagggttct caggatggg gaggggaccc aaggctcttg tggctatcca
3001 gaaaggagac tggggcccat ccagggccat gggacgtacc tttaccgtt cactgctgga
3061 agatgattga atgggtggcc tggtagggc tggagagtat cgggtggccc agcagggtat
3121 cttctcccg gacccctacc caggccattg gaggacagcc catagagagg accggagctc
3181 gtcacccagg agtggggttg gtcgagctgac actggggctg gaggccctgg aggaagggcc
3241 cccagatctt ccactggctc cctcgggagg ggttgggtcc gatggtcttg atggtgacct
3301 ggcggtgggg gbaacgaaa ggcctgggag cctctctca caggacctca ggcctctaca
3361 ggggttgggg gagggaagcc cactgctctt ggcctccagg actgagggct atgttggctc
3421 gacggctggg agcccccagg ccgagctatg gaaacccatc ggggttggg cttagacctc
3481 tttgacccca gggggctctc tggctcctgt cgggcttggc ggtgctgctc tagaagagcc
3541 caagatcttc tcttctggga agaatggggt tgtcgaagac gtttctgctt cgggggtg
3601 tatgagagac cctggaatac caatccggag agaaaggcat gctctccggc cccaccttc
3661 tccgagcttc agccagggct tggagcaact ctatcactgg gaccaggaat catcggagca
3721 ggggctctcc ccaggtaac ttgaaaggag ccccaactga gaggaccttg agtaacctag
3781 cctggggtga cctggtatgag aggtggtgag acgtccgttg ctcttagagt ggggaaaggcc
3841 tggcttcttg tctcctggc taccagagag gggaggggtc ctctgggac attacattca
3901 gggggagctg gctacgggg aaactggccc gagggaactt tccctgctgc tggaa

```

Herceptin Binding by Direct Elisa 10/5/99

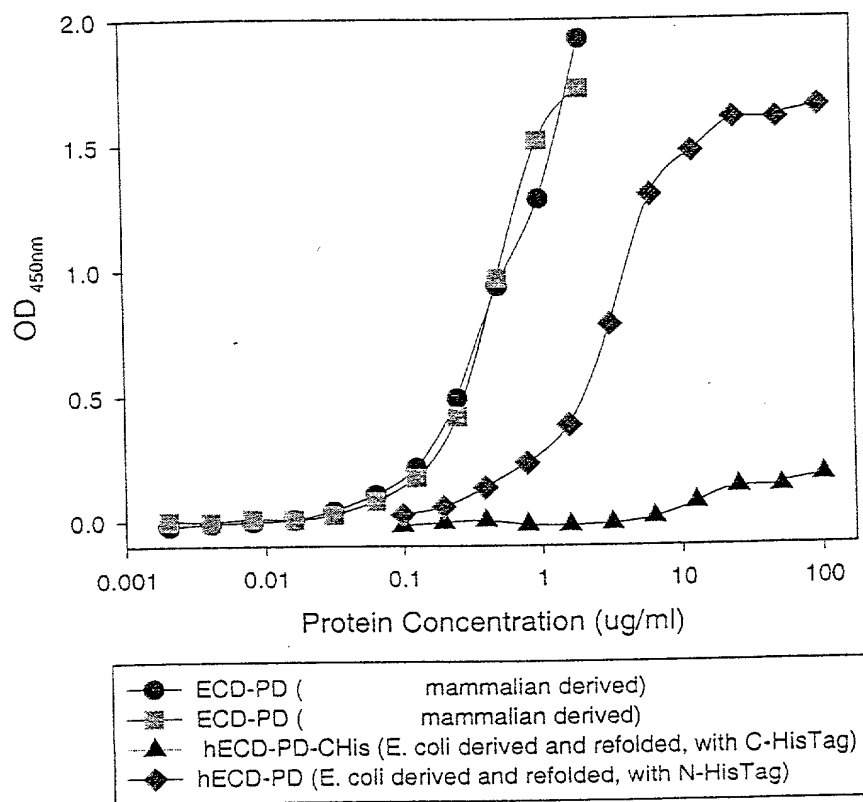
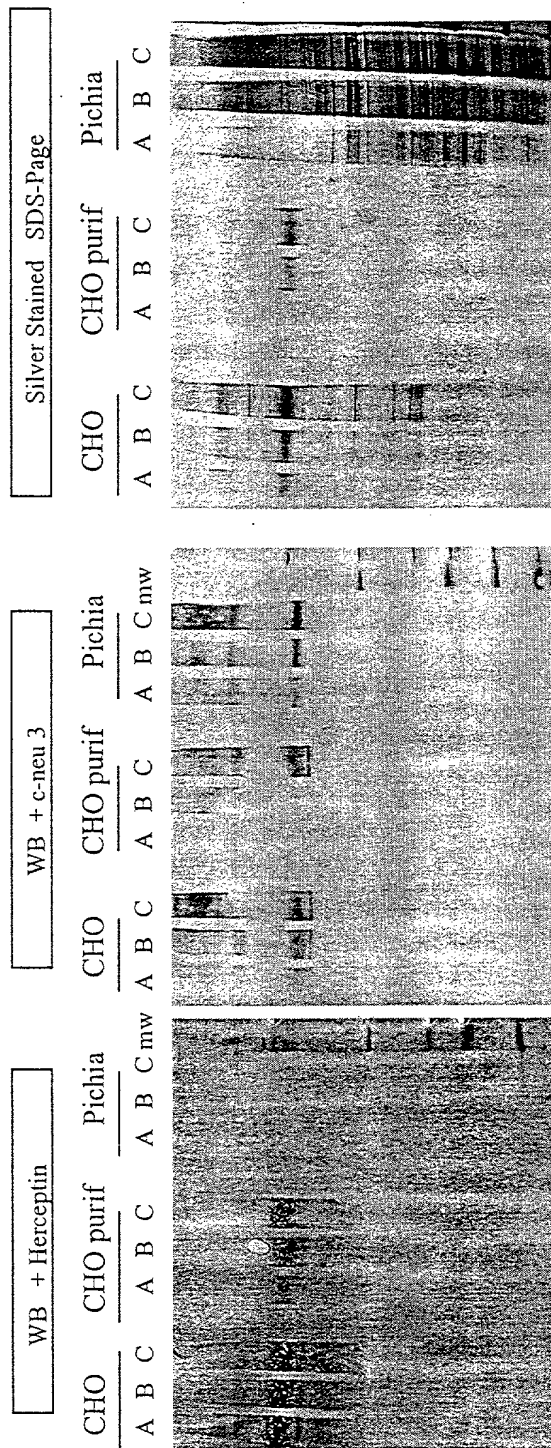


Fig. 17

Comparison of Her2neu ECD-PD Expression in CHO-K1 (S/SF) and Pichia (Non reducing conditions)



Legend : CHO; A, B, C = 2,5µl / 5µl / 10µl

CHO purif; A, B, C = 125ng / 250ng / 500ng

Pichia ; A, B, C = 2,5µl / 5µl / 10µl from a 1/30 dilution of OD 120

Fig. 19 (SEQ ID NO:11)

| | | | | | | |
|-------------|-------------|-------------|-------------|-------------|-------------|------|
| atggagctgg | cggcctgggtg | cggttggggg | ttctctctcg | ccctcctgtc | ccccggagcc | 60 |
| gcgggtaccc | aagtgtgtac | cggtagcgac | atgaagttgc | gactccctgc | cagtcctgag | 120 |
| acccacctgg | acatgcttcg | ccacctctac | cagggctgtc | aggtgggtgca | gggcaatttg | 180 |
| gagcttacct | acctgcccgc | caatgccagc | ctctcattcc | tgcaggacat | ccaggaagtc | 240 |
| cagggataca | tgctcatcgc | tcacaaccga | gtgaaacacg | tcccactgca | gaggttgccg | 300 |
| atcgtgagag | ggactcagct | ctttgaggac | aagtatgccc | tggctgtgct | agacaaccga | 360 |
| gaccctttgg | acaacgtcac | caccgcgcgc | ccaggcagaa | cccagaagg | gctgcgggag | 420 |
| ctgcagcttc | gaagtctcac | agagatcttg | aaggatgtcc | ttttgatccg | tgggaaccct | 480 |
| cagctctgct | accaggacat | ggttttgtgg | aaggatgtcc | tccgtaagaa | taaccagctg | 540 |
| gtcctgtcgc | acatggacac | caatcgttcc | cgggcctgtc | caccttgtgc | cccaacctgc | 600 |
| aaagacaatc | actgttgggg | tgagagtcc | gaagactgtc | agatcttgac | tggcaccatc | 660 |
| tgtactagt | gctgtgccc | gtgcaagggc | cggctgccc | ctgactgttg | ccatgagcag | 720 |
| tgtgctgcag | gctgcacggg | tcccaagcat | tctgactgcc | tggcctgcct | ccacttcaat | 780 |
| catagtggta | tctgtgagct | gcactgccc | gcccctcatca | cctacaacac | agacaccttc | 840 |
| gagtcctatgc | tcaaccctga | gggtcgctac | acctttgggtg | ccagctgtgt | gaccacctgc | 900 |
| ccctacaact | acctctccac | ggaagtggga | tccctgcactc | tggctgtgtcc | cccgaacaac | 960 |
| caagaggtca | cagctgagga | cggaacacag | cgggtgtgaga | aatgcagcaa | gccctgtgct | 1020 |
| ggagtatgct | atggtctggg | catggagcac | ctccgagggg | cgagggccat | caccagtgc | 1080 |
| aatatccagg | agtttgctgg | ctgcaagaag | atctttggga | gcctggcatt | tttgccggag | 1140 |
| agctttgatg | ggaacccctc | ctccggcgtt | gccccactga | agccagagca | tctccaagt | 1200 |
| ttcgaaaccc | tggaggagat | cacagggttac | ctatacatctt | cagcatggcc | agagagcttc | 1260 |
| caagacctca | gtgtcttcca | gaaccttcgg | gtcattcggg | gacggattct | ccatgatggt | 1320 |
| gcttactcat | tgacgttgca | aggcctgggg | attcactcac | tggggctacg | ctcactgcgg | 1380 |
| gagctgggca | gtggattggc | tctcattcac | cgcaacaccc | atctctgctt | tgtaaacact | 1440 |
| gtaccttggg | accagctctt | ccggaacccg | caccaggccc | tactccacag | tgggaaccgg | 1500 |
| ccagaagagg | catgtgggtct | tgaggggctt | gtctgtaact | cactgtgtgc | ccgtggggc | 1560 |
| tgctgggggg | cagggccccc | ccagtgtgtc | aactgcagtc | agttcctccg | ggggccaggag | 1620 |
| tgtgtggagg | agtgcgcagt | atggaagggg | ctccccagg | agtatgtgag | gggcaagcac | 1680 |
| tgtctgccat | gccaccccga | gtgtcagcct | caaaacagct | cggagacctg | ctatggatcg | 1740 |
| gaggctgacc | agtgtgaggc | ttgtgcccac | tacaaggact | catcttcctg | tgtggctcgc | 1800 |
| tgccccagtg | gtgtgaagcc | agacctctcc | tacatgccta | tctggaagta | cccggatgag | 1860 |
| gagggcata | gtcagccatg | ccccatcaac | tgcacccact | catgtgtgga | cctggacgaa | 1920 |
| cgaggctgcc | cagcagagca | gagagccagc | ccagtgcacat | tcatcattgc | aactgtgggtg | 1980 |
| ggcgtcctgt | tgttcctgat | catagtgggtg | gtcattggaa | tcctaataca | acgaaggcca | 2040 |
| cagaagatcc | ggaagtatac | catgcgtagg | ctgctgcagg | agaccgagct | ggtggagccg | 2100 |
| ctgacgccc | gtggagctgt | gcccacaccg | gctcagatgc | ggatccctaaa | ggagacagag | 2160 |
| ctaaggaagc | tgaaggtgct | tgggtcagga | gccttcggca | ctgtctacaa | gggcatctgg | 2220 |
| atcccagatg | gggagaacgt | gaaaatcccc | gtggccatca | aggtgttgag | ggaaaacaca | 2280 |
| tctcctaaa | ctaacaaaga | aatcctagat | gaagcgtacg | tcatggctgg | tgtgggttct | 2340 |
| ccatatgtgt | cccgcctcct | gggcattctgc | ctgacatcca | cagtgcagct | ggtgacacag | 2400 |
| cttatgccct | atggctgcct | tctggaccat | gtccgagaac | accgaggtcg | cttaggctcc | 2460 |
| caggacctgc | tcaactgggtg | tgttcagatt | gccaaagggga | tgagctacct | ggaggaagtt | 2520 |
| cggcttggtc | acagggacct | agctgcccga | aacgtgctag | tcaagagtcc | caaccacgtc | 2580 |
| aagattaccg | acttcgggct | ggcaocggctg | ctggacattg | atgagactga | ataccatgca | 2640 |
| gatgggggca | aggtgcccac | caagtggatg | gcattggaat | ctattctcag | acgccggttc | 2700 |
| actcatcaga | gtgatgtgtg | gagctatggt | gtgactgtgt | gggagctgat | gacctttggg | 2760 |
| gccaacacct | acgatgggat | cccagctcgg | gagatccctg | atttgctgga | gaaggagaa | 2820 |
| cgcctacctc | agcctccaat | ctgcaccatc | gacgtctaca | tgatcatggg | caaatgttgg | 2880 |
| atgattgact | ccgaatgtcg | cccagatttc | cgggagttgg | tatcagaatt | ctcccgtatg | 2940 |
| gcaagggacc | cccagcgctt | tgtggtcatc | cagaacgagg | acttaggccc | ctccagcccc | 3000 |
| atggacagca | ccttctaccg | ttcactgctg | gaggatgatg | acatggggga | gctggctcat | 3060 |
| gctgaagagt | acctggtacc | ccagcaggga | ttcttctccc | cagaccctgc | cctaggtaact | 3120 |
| gggagcacag | cccaccgcag | acaccgcagc | tcgtcgggca | ggagtggcgg | tgggtgagctg | 3180 |
| acactggggc | tggagccctc | ggaagaagag | ccccccagat | ctccactggc | tccctccgaa | 3240 |
| ggggctggct | ccgatgtgtt | tgatgggtgac | ctggcagttg | gggtaaccaa | aggactgcag | 3300 |
| agcctctctc | cacatgacct | cagccctcta | cagcgggtaca | gtgaggatcc | cacattacct | 3360 |

Fig. 19 (SEQ ID NO:11)

| | | | | | | |
|------------|-------------|------------|------------|-------------|------------|------|
| ctgcccccg | agactgatgg | ctacgttgct | cccctggcct | gcagcccca | gcccgagtat | 3420 |
| gtgaaccagc | cagagggttcg | gcctcagtct | cccttgaccc | cagaggggtcc | tccgcctccc | 3480 |
| atccgacctg | ctggtgctac | tctagaaaga | ccaagactc | tctctcctgg | gaaaaatggg | 3540 |
| gttgtaaag | acgtttttgc | ctttgggggt | gctgtggaga | accctgaata | cctagcacc | 3600 |
| agagcaggca | ctgcctotca | gccccaccct | tctcctgcct | tcagcccagc | ctttgacaac | 3660 |
| ctctattact | gggaccagaa | ctcatcggag | cagggtcctc | caccaagtac | ctttgaagg | 3720 |
| acccccactg | cagagaacc | tgagtaccta | ggcctggatg | tgccagtatg | a | 3771 |

CTGCCCCCG AGACTGATGG CTACGTTGCT CCCCTGGCCT GCAGCCCCA GCCCGAGTAT 3420

Fig. 20 (SEQ ID NO:14)

```

Met Glu Leu Ala Ala Trp Cys Arg Trp Gly Phe Leu Leu Ala Leu Leu
 1          5          10          15
Ser Pro Gly Ala Ala Gly Thr Gln Val Cys Thr Gly Thr Asp Met Lys
          20          25          30
Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
          35          40          45
Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
          50          55          60
Leu Pro Ala Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
65          70          75          80
Gln Gly Tyr Met Leu Ile Ala His Asn Arg Val Lys His Val Pro Leu
          85          90          95
Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Lys Tyr
          100          105          110
Ala Leu Ala Val Leu Asp Asn Arg Asp Pro Leu Asp Asn Val Thr Thr
          115          120          125
Ala Ala Pro Gly Arg Thr Pro Glu Gly Leu Arg Glu Leu Gln Leu Arg
          130          135          140
Ser Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Arg Gly Asn Pro
145          150          155          160
Gln Leu Cys Tyr Gln Asp Met Val Leu Trp Lys Asp Val Leu Arg Lys
          165          170          175
Asn Asn Gln Leu Ala Pro Val Asp Met Asp Thr Asn Arg Ser Arg Ala
          180          185          190
Cys Pro Pro Cys Ala Pro Thr Cys Lys Asp Asn His Cys Trp Gly Glu
          195          200          205
Ser Pro Glu Asp Cys Gln Ile Leu Thr Gly Thr Ile Cys Thr Ser Gly
          210          215          220
Cys Ala Arg Cys Lys Gly Arg Leu Pro Thr Asp Cys Cys His Glu Gln
225          230          235          240
Cys Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys
          245          250          255
Leu His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu
          260          265          270
Ile Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Leu Asn Pro Glu Gly
          275          280          285
Arg Tyr Thr Phe Gly Ala Ser Cys Val Thr Thr Cys Pro Tyr Asn Tyr
          290          295          300
Leu Ser Thr Glu Val Gly Ser Cys Thr Leu Val Cys Pro Pro Asn Asn
305          310          315          320
Gln Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser
          325          330          335
Lys Pro Cys Ala Gly Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg
          340          345          350
Gly Ala Arg Ala Ile Thr Ser Asp Asn Ile Gln Glu Phe Ala Gly Cys
          355          360          365
Lys Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly
          370          375          380
Asn Pro Ser Ser Gly Val Ala Pro Leu Lys Pro Glu His Leu Gln Val
385          390          395          400
Phe Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp
          405          410          415
Pro Glu Ser Phe Gln Asp Leu Ser Val Phe Gln Asn Leu Arg Val Ile

```


Fig. 20 (SEQ ID NO:14)

Asp Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu
 885 890 895
 Arg Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr
 900 905 910
 Val Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro
 915 920 925
 Ala Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln
 930 935 940
 Pro Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp
 945 950 955 960
 Met Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu
 965 970 975
 Phe Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn
 980 985 990
 Glu Asp Leu Gly Pro Ser Ser Pro Met Asp Ser Thr Phe Tyr Arg Ser
 995 1000 1005
 Leu Leu Glu Asp Asp Asp Met Gly Glu Leu Val Asp Ala Glu Glu Tyr
 1010 1015 1020
 Leu Val Pro Gln Gln Gly Phe Phe Ser Pro Asp Pro Ala Leu Gly Thr
 1025 1030 1035 1040
 Gly Ser Thr Ala His Arg Arg His Arg Ser Ser Ser Ala Arg Ser Gly
 1045 1050 1055
 Gly Gly Glu Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Pro Pro
 1060 1065 1070
 Arg Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp
 1075 1080 1085
 Gly Asp Leu Ala Val Gly Val Thr Lys Gly Leu Gln Ser Leu Ser Pro
 1090 1095 1100
 His Asp Leu Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Leu Pro
 1105 1110 1115 1120
 Leu Pro Pro Glu Thr Asp Gly Tyr Val Ala Pro Leu Ala Cys Ser Pro
 1125 1130 1135
 Gln Pro Glu Tyr Val Asn Gln Pro Glu Val Arg Pro Gln Ser Pro Leu
 1140 1145 1150
 Thr Pro Glu Gly Pro Pro Pro Pro Ile Arg Pro Ala Gly Ala Thr Leu
 1155 1160 1165
 Glu Arg Pro Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp
 1170 1175 1180
 Val Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Ala Pro
 1185 1190 1195 1200
 Arg Ala Gly Thr Ala Ser Gln Pro His Pro Ser Pro Ala Phe Ser Pro
 1205 1210 1215
 Ala Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asn Ser Ser Glu Gln Gly
 1220 1225 1230
 Pro Pro Pro Ser Thr Phe Glu Gly Thr Pro Thr Ala Glu Asn Pro Glu
 1235 1240 1245
 Tyr Leu Gly Leu Asp Val Pro Val
 1250 1255

DECLARATION

As a below named inventor, I declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **HER-2/NEU FUSION PROTEINS** the specification of which _____ is attached hereto or **X** was filed on _____ as Application No. _____ and was amended on _____ (if applicable).

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56. I claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

| Application No. | Filing Date |
|-----------------|-------------|
| 60/117,976 | 01/29/99 |

I claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| Application No. | Date of Filing | Status |
|-----------------|----------------|--------|
| | | |

| | | | |
|--------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------|
| Full Name of Inventor 1: | Last Name: CHEEVER | First Name: MARTIN | Middle Name or Initial: A. |
| Residence & Citizenship: | City: Mercer Island | State/Foreign Country: Washington | Country of Citizenship: USA |
| Post Office Address: | Post Office Address: 6210 SE 22nd | City: Seattle | State/Country: Washington |
| | | | Postal Code: 98040 |

| | | | |
|--------------------------|------------------------------------------------------|------------------------------------------|-------------------------------------------|
| Full Name of Inventor 2: | Last Name: GHEYSEN | First Name: DIRK | Middle Name or Initial: |
| Residence & Citizenship: | City: Rixensart | State/Foreign Country: Belgium | Country of Citizenship: BELGIUM |
| Post Office Address: | Post Office Address: 89, rue de l'Institut | City: Rixensart | State/Country: Belgium |
| | | | Postal Code: B-1330 |

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1

Martin A. Cheever

Date

Signature of Inventor 2

Dirk Gheysen

Date

SF 1063331 v1